

Risks and benefits of dual-use research

Negotiations over a sensitive scientific publication that could be misused by bioterrorists highlight trouble ahead unless appropriate guidelines are developed.

Scientists, security experts and journals have done a great deal to face up to the risk of bioterrorism, but there is still considerable uncertainty over how to handle 'dual use' research with outcomes that might be used to do harm (see page 860). This fact is underscored by confusion about a paper on such research that was accepted for publication last month by the *Proceedings of the National Academy of Sciences* (PNAS).

The episode began on 25 May, when the journal announced that it would publish a paper by Lawrence Wein, a professor of management at the Stanford University Graduate School of Business, California. Working with a graduate student, Wein had constructed a model of a bioterrorist attack on the US milk supply. Stewart Simonson, assistant secretary for public health emergency preparedness at the US Department of Health and Human Services (HHS), was shown the paper by a reporter, who had obtained a copy under embargo. He promptly asked the National Academy of Sciences, which publishes the journal, not to release it.

Simonson's concern, which was shared by other officials, was that details of the paper could be helpful to terrorists. The National Academy agreed to delay publication of the paper and met with Simonson and other HHS officials on 7 June. As *Nature* went to press, the National Academy had not announced how it would move forward, but seemed inclined to publish the paper essentially unmodified.

Both Wein and the journal were well aware of the sensitive nature of this paper. In fact, Wein briefed Simonson's office on the research last autumn, and Simonson says that he conveyed his concerns about the work to Wein at that time. Wein contradicts that statement, maintaining that the HHS never replied to his briefing. When Wein submitted his paper to PNAS, the journal sent it through two layers of review, as specified by many journals, including *Nature*, in 2003 — one for scientific accuracy and one for biosecurity. None of the reviewers opposed publication, and the editors concluded

that the paper's potential to inform biosecurity efforts outweighed the risk of it giving a blueprint to terrorists. So the journal decided to publish.

Each of the parties to this dispute could have acted differently. For instance, the apparent breakdown in initial communication between Wein and the HHS implies a cursory approach to such a sensitive matter on both sides and a lack of a robust system for such alerts by responsible researchers.

The HHS did not fund Wein's work, and has never intervened in a publication in this fashion before. In doing so, however, it has raised the profile of the issue. The department might have accomplished more by working behind the scenes with Wein and the dairy industry to increase safety, instead of taking action against a journal. Much of the information in Wein's paper is readily available on the Internet anyway.

PNAS followed its normal procedures but was prudent to allow a brief delay to listen to the HHS. Having previously committed to the paper, in the absence of any significant new security risks being raised, it should now stick to its decision. But there will doubtless follow a broader debate on whether such papers should be submitted to, and accepted by, a high-profile scientific journal.

The greatest concern is in the need for clarity. It is important to develop clear guidelines about what research is considered sensitive, what is expected of researchers whose work produces dual-use outcomes, and how the government should in practice respond without losing the priceless virtues of open scientific scrutiny. Without such clarity, officials insensitive to those virtues may institute precautionary measures that reach far beyond what is appropriate. The US National Science Advisory Board for Biosecurity, which was set up a year ago by the US government to address such concerns, will hold its first meeting at the end of this month, and will need to act promptly. ■

Save the people, too

Conservationists must pay attention to the needs of local human, as well as animal, populations.

To have real passion for one's work is a wonderful thing. And there are few people more passionate than the biologists who strive to preserve biodiversity across the developing world. Many are prepared endure physical privations, infectious diseases, low pay and threats of violence, all in the name of conservation.

But passion can sometimes distort judgment. Just as starry-eyed lovers may be blind to one another's faults, a true believer in any cause can ignore uncomfortable facts that conflict with its goals. That is

why the motivations and actions of conservation biologists who are working in Myanmar, with the blessing of its brutal military regime, merit close scrutiny.

In the past, such scrutiny has been uncomfortable for some of the individuals concerned — most notably following the 1997 publication of an article in *The Observer*, a UK newspaper, entitled 'Save the rhino, kill the people'. This linked such venerable bodies as the Smithsonian Institution in Washington DC and the Wildlife Conservation Society, based in New York, with abuses of human rights in the southeast Asian nation formerly known as Burma.

The biologists who were singled out for criticism in that article argue, with good cause, that it misrepresented their efforts. And it is apparent from a News Feature on page 870 of this issue that they are working with clear consciences, despite having to engage on



some level with the military regime if they are to achieve their goals.

Yet it is important to ask whether the distorting lens of passion has come into play. The imminent threat to Myanmar's biodiversity is not in doubt, nor is the desire of Burmese conservationists for foreign assistance. It is also good to hear that some biologists working in Myanmar have sought the views of ordinary Burmese people.

But some statements do give cause for concern. Attempts to justify engaging with a government guilty of atrocities by arguing that other regimes are just as bad are not compelling. The suggestion that Burmese exiles have exaggerated the abuses in Myanmar is discomfiting, as is the notion that conservation biologists need to use 'charm and guile' to convince suspicious politicians back in the United States that they are not abetting the Burmese junta.

These may just be poorly chosen words. But it is hard not to wonder, on hearing the stories of those working in Myanmar, whether some conservation biologists are prone to rush to the aid of threatened biota first, and to wrestle with the wider political and humanitarian implications only later. If that's the case, it is a dangerous tendency. As any psychologist will tell you, the human mind is adept at conjuring up post hoc justifications for a course of action that has already been decided.

We should also heed the lessons of history. Today it is widely accepted that effective conservation requires the involvement of local

people, and should bring them tangible benefits. But the annals of conservation are littered with instances of people being seen as obstacles that must be removed to make way for parks and reserves. This isn't even limited to undemocratic countries: in the United States, decades ago, conservationists pursued projects such as the Shenandoah National Park in Virginia, whose creators portrayed local mountain farmers as backward and hounded them off their land.

Given this legacy, conservation biologists have a responsibility to ensure that their efforts do not conflict with local peoples' rights, or lend legitimacy to regimes that have dismal human-rights records. This doesn't mean that they shouldn't work at all in countries such as Myanmar. But they should set out for their field sites with their eyes wide open, having researched the humanitarian issues and engaged with parties who may not share their view that conserving biodiversity is the overwhelming priority for the region in question. That will build more confidence that saving the rhino doesn't require unacceptable compromises on human rights. ■

"Conservation biologists have a responsibility to ensure that their efforts do not conflict with local people's rights or lend legitimacy to regimes that have dismal human-rights records."

Much whaling and gnashing of teeth

The International Whaling Commission may be messy, but it's the only game in town.

Marine biologists at this month's annual meeting of the International Whaling Commission (IWC) at Ulsan in South Korea will require considerable patience and fortitude. "It's like banging your head against the wall," complained one scientist there for the preliminary scientific-committee meeting.

The main bone of contention at this year's meeting is a proposal by Japan to double the scope of its 'research whaling' programme — its thinly disguised arrangement to continue some whaling despite a moratorium on commercial whaling that the IWC implemented in 1986 (see *Nature* 435, 550; 2005). The plan may get a sympathetic hearing at Ulsan because pro-whaling nations now seem to have a majority on the IWC for the first time (see page 861). This has come about because 23 new members — some with a dubious interest in whales, dead or alive — have joined the IWC in the past five years, taking its total membership to 62. Whaling opponents whisper that Japan "goes shopping", as one of them puts it, for small, poor countries such as Kiribati and Tuvalu in the south Pacific to join the body in exchange for aid.

It is unlikely, however, that the new composition of the IWC will lead to radical changes in whaling rules, which would require a three-quarters majority. Japan's research programme was never actually approved by the body in the first place; Japan has sometimes chosen to brush the IWC's non-binding views on the matter aside.

"Resolutions adopted by the IWC against Japan's whale research programmes are political statements that have nothing to do with science," sniffed Joji Morishita, a spokesman for Japan's fisheries agency, in a statement issued during the meeting.

Opponents of whaling continue to regard Japan's research programme as an affront to conservation efforts, and Japan has been hard-pressed to come up with convincing, peer-reviewed articles supporting it. Now it says it needs an even larger programme to address the demands of the IWC's scientific committee. More sophisticated analysis requires a greater sample size — who can argue with that?

Given all this chicanery, one might be tempted to ask why researchers should bother to spend so many long days and nights in South Korea engaging in the IWC process.

But buffeted by criticism as it may be, the IWC continues to implement the international regime that stands in the way of unregulated whaling — and of the probable extinction of several whale species. Before the moratorium, Japan's yearly quota of minke whale in the Southern Hemisphere was 1,941; under its proposed research programme, it would catch 935.

And despite its grouching, Japan wants to be seen as a good international citizen; it is unlikely to pack up its marbles and go home. It will remain at the table, infuriating its opponents at times but basically conforming with an imperfect international process. Conservation biologists should do likewise, cajoling more friendly nations to sign on and grimly adhering to the only path that can, in its convoluted way, save the whales. ■

"The IWC continues to implement the international regime that stands in the way of unregulated whaling."

RESEARCH HIGHLIGHTS

Culture shock

Nature Med. doi:10.1038/nm1268 (2005); *Proc. Natl Acad. Sci. USA* doi:10.1073/pnas.0503596102 (2005); *Science* doi:10.1126/science.1114016 (2005)

More than 170 million people worldwide are infected with the virus that causes hepatitis C, a major liver disease. Understanding the life cycle of the virus (pictured) is essential for developing effective treatments, but progress has been limited because the virus is difficult to grow in culture and therefore hard to study.

In independent papers, three teams now report successful *in vitro* systems for propagation, using a unique hepatitis C virus clone derived from a Japanese patient by Takaji Wakita at the Tokyo Metropolitan Institute for Neuroscience. His group, and teams headed by Francis Chisari at the Scripps Research Institute, California, and Charles Rice at Rockefeller University, New York, propagated this clone in a human liver-cancer cell line. Their systems produce high yields of virus that can be used to infect further cells.

IMAGE
UNAVAILABLE
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REASONS

ARTIST'S IMPRESSION: R. KIGHTLEY/SPL

MATERIALS

Foul play

J. Am. Chem. Soc. **127**, 7972–7973 (2005)

Mussels stick tenaciously to the hulls of boats, and this increases the boats' drag. But Phillip Messersmith and colleagues at Northwestern University in Illinois, Evanston, plan to turn one of their own proteins against them.

They have developed an antifouling polymer that prevents biological adhesion — a non-stick compound is anchored to a surface by a peptide that mimics the adhesive protein of blue mussels (*Mytilus edulis*, pictured). The non-stick element is a polymer made from an artificial analogue of glycine.

The material could have applications not only in marine engineering but also in medicine, for example, to keep implanted

devices clean. Titanium coated with the antifouling polymer remains relatively cell-free in a culture of tissue-forming fibroblasts for months.

IMMUNOLOGY

Poxy antibodies

Nature Med. doi:10.1038/nm1261 (2005)

More than 200 years after Edward Jenner realized that infection with cowpox made milkmaids resistant to the smallpox virus, a group led by Genoveffa Franchini from the National Cancer Institute in Bethesda, Maryland, has discovered how a smallpox vaccine based on the *vaccinia* virus confers immunity.

Using macaques infected with monkeypox virus, which is a good model for smallpox infection in humans, the researchers showed that the protective power of the vaccine is mediated by the immune system's B cells, rather than its T cells. The B cells produce antibodies that bind specific poxvirus proteins — and the researchers found that antibodies from vaccinated humans protected macaques from severe disease. The finding may assist the search for a safer alternative to the current live-virus vaccine.

NANOTECHNOLOGY

Going for gold

J. Am. Chem. Soc. doi:10.1021/ja042621o (2005)
Spherical cages of carbon atoms that attach gently to gold should allow more complex molecular patterning of gold surfaces,

according to Paul Weiss's group at the Pennsylvania State University, University Park.

His team persuaded 1-Adamantanethiol molecules to self-assemble into a monolayer on gold. Because these molecules' interactions are weak, they can be displaced by other molecules that bind to gold, such as alkanethiols. This should make it possible to print patterns of molecules, perhaps with conducting or sensing properties, into the 1-Adamantanethiolate layer. The surrounding layer would prevent the pattern spreading by diffusion, overcoming a problem encountered when some molecules are printed on bare gold surfaces.

MEDICINE

Barrier grief

J. Clin. Invest. **115**, 1607–1615 (2005)

To cause meningitis, *Streptococcus pneumoniae* must find its way across the blood–brain barrier. A group led by Jörg Weber of the Charité Medical School in Berlin, Germany, has now worked out how the bacterium damages the endothelial cells that make up the barrier. They find that the pathogen induces programmed cell death through two different mechanisms. One pathway is triggered by toxins produced by living *S. pneumoniae*, the other by components of its cell wall. The latter mechanism has implications for therapeutic treatment — antibiotics that target the *S. pneumoniae* cell wall might cause further tissue damage through the release of cell-wall debris.

IMAGE
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REASONS

R. HODDINOTT/NATUREPL.COM

CELL BIOLOGY

Calling tails

J. Cell Biol. doi:10.1083/jcb.200411001 (2005)
Eggs attract sperm by releasing chemicals that boost calcium ion (Ca^{2+}) levels inside a sperm, so altering the direction in which it swims. But rather than responding to overall levels of Ca^{2+} , as previously thought, the sperm react to the rate at which its concentration changes, reports a team led by Christopher Wood at the National Autonomous University of Mexico, Cuernavaca.

Experiments in sperm from the sea urchin *Arbacia punctulata* suggest that attractant chemicals trigger two waves of Ca^{2+} that pass through the sperm's tail. The first is short and rapid, the second long and slow. Blocking the first rapid flux stops the sperm changing direction even though the second wave elevates Ca^{2+} levels, revealing a surprising complexity in calcium-ion signalling.

QUANTUM PHYSICS

Lamb chops

Phys. Rev Lett. **94**, 223001 (2005)

The field theory of electromagnetism — known as quantum electrodynamics — is the best-tested theory in physics. But researchers will not rest until they have tested its validity in extremely strong electric fields, such as those generated by heavy nuclei.

To do this, Alexandre Gumberidze of the Heavy Ion Research Centre in Darmstadt, Germany, and his colleagues measured a tiny split in the energy levels of a uranium ion from which all but one electron had been stripped. This made it possible to see the Lamb shift, a split in energy levels usually visible only in the single-electron hydrogen atom. The test was three times more precise than the previous best measurement.

IMAGE
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VIRAL TRANSPORT

Stowaways

Nature Cell Biol. doi:10.1038/ncb1269 (2005)

Certain viruses improve their ability to infect cells by stowing away in structures called internal vesicles, according to a team led by Jean Gruenberg at the University of Geneva, Switzerland. This means that infection takes place in two steps — not one, as previously thought.

The team tracked the vesicular stomatitis virus as it infected cultured cells. The cell swallowed the virus into a structure called an early endosome at the cell's edge, as expected. But instead of escaping straight from the endosome into the outer regions of the cell, the virus within its endosome entered a small bubble-like vesicle. These vesicles only fuse with the outer membrane of the endosome when the complex is deep inside the cell, releasing the virus right next to the cell's nucleus.

WEST NILE DISEASE

Blood wedding

Proc. Natl Acad. Sci. USA

doi:10.1073/pnas.0503835102 (2005)

The discovery that one mosquito can transmit the West Nile virus directly to another may help explain the surprisingly rapid spread of the disease through North America.

Usually mosquitoes (*Culex pipiens quinquefasciatus*) pick up the West Nile virus by feeding on birds infected with it. But researchers led by Stephen Higgs of the University of Texas Medical Branch in Galveston have shown that the virus can pass between two mosquitoes if they sip blood simultaneously from an uninfected host. This type of transmission has previously been demonstrated in ticks and blackflies. In this case, the recipient mosquito may acquire the virus by directly ingesting infected saliva from a feeding neighbour, but this remains to be proven.

DATA STORAGE

Pillar talk

J. Appl. Phys. **97**, 103910 (2005)

One way to pack more data on to magnetic disks is to pattern the surface, defining small dots that store single bits. Another approach is to use multiple magnetic layers, so more than one bit can be stored per spot.

Combining the two tactics, researchers from the University of Konstanz in Germany and the Hitachi San Jose Research Center in California deposited multiple layers of cobalt and palladium onto a field of silicon pillars, spaced 300 nanometres apart. They stored two bits per pillar, giving higher data densities than otherwise possible with this scale of patterning.

JOURNAL CLUB

John Brookfield
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A population geneticist ponders the evolution of his field.

Since I started in the business of population genetics, the field has been transformed by the extraordinary increase in size of the data sets. A recent paper in *Science* (D. A. Hinds *et al.* **307**, 1072–1079; 2005) is a prime example of such a set, but we have yet to exploit them.

In the 1970s, studies followed a common pattern. First they

identified a gene with different variants in a population. That meant looking for detectable differences between individuals — for example, as in my PhD, differences in the charges of enzymes. Once a variation in a gene, known as a polymorphism, was identified, they tried to establish whether natural selection was operating on it.

Now that we have DNA sequences, we can find single nucleotide polymorphisms, in which a single letter of the

sequence varies. The authors of the *Science* paper use DNA chips to find the frequencies of more than 1.5 million single nucleotide polymorphisms in three human populations. But how can we use such a wealth of figures to find out about natural selection?

One tantalizing idea is that we can measure — in an objective way — how quickly species are evolving. Genetic differences in a population accumulate randomly over time, so regions of the genome where polymorphisms are rare

must have been swept clean recently by a spreading variant of a gene. These sweeps are evidence of adaptive change.

Counting the number of sweeps that have affected the human genome will, I suspect, tell us that our species is changing rapidly through adaptation. That could explain why we seem so different from our ape relatives.

We might also identify the genes in which these changes have occurred — a perennial goal of human evolutionary genetics.

NEWS



BRAND X PICTURES

Biologists asked to breed a culture of responsibility in face of terrorism

Diplomats and biosecurity experts meeting in Geneva this week are urging life scientists to act responsibly and prevent potential misuse of their work.

Governments can enact laws to prosecute people who use science to scare or hurt the public. And they can make rules to promote safety in research. But laws and rules go only so far in heading off potential abuses of science. When it comes to weighing up the potential risks and benefits of a piece of research, or deciding whether to publish a controversial result, scientists must fill the gap by adopting their own principles for proper conduct, say those at the Geneva conference.

The meeting, which runs from 13 to 24 June, focuses on codes of conduct in life-sciences research. It is the third 'Meeting of Experts' — a series of conferences intended to promote the international treaty that bans biological weapons. Formal negotiations on how to enforce the treaty collapsed in 2001, but are scheduled to resume next year.

Unlike physicists, who were forced to face up to the potential consequences of their work when nuclear weapons were developed in the 1940s, many biologists still do not believe that their work could possibly be misused, say biosecurity experts. But several recent papers have highlighted how bona fide research could be abused by terrorists or governments developing biological weapons.

For example, in 2001 an Australian team accidentally created a deadly version of

mousepox, a virus that is related to smallpox, by removing a single gene (R. J. Jackson *et al.* *J. Virol.* **75**, 1205–1210; 2001). And in 2002, the journal *Science* published a paper describing how researchers had synthesized a whole polio genome from scratch (J. Cello, A. V. Paul and E. Wimmer *Science* **297**, 1016–1018; 2002). Scientists played down the novelty of the paper, but alarm among the public was so great that a member of US Congress criticized the work and asked the US executive branch to review policies intended to stop research being used by terrorists (see *Nature* **418**, 265; 2002).

The answers to such problems, say experts at the Geneva meeting, are codes of conduct. These are statements by scientific societies, trade organizations or other bodies that lay out principles to govern their members' activities. Advocates say they would force each researcher to think about the proper conduct, dissemination and use of his or her work.

"Developing a code of conduct really would give structure to the awareness that we need to create, especially among young professionals," says Mark Smolinski of the Nuclear Threat Initiative, an arms control think-tank based in Washington DC. He and others want to see scientists take an active role in preventing potential abuses of their work.

For instance, a researcher might choose to brief key government officials about a sensitive finding instead of publishing it in a widely

distributed journal — a possibility raised in ongoing controversy over a paper that models a bioterrorist attack on the US milk supply. Publication of this study has been delayed after protest from the US government (see page 855).

Some life-sciences organizations, such as the Washington-based American Society for Microbiology and the Australian Society for Microbiology in Melbourne, have already adopted codes of ethics that refer to biological weapons. But many experts feel that more researchers should adopt codes, especially at local levels. An influential report issued in 2003 by the US National Academy of Sciences said that awareness of potential misuse of life sciences "varies widely" among researchers. It advised more education.

Just last week, a report was issued by an expert US commission, chaired by Harold Brown, former US secretary of defence, and Nobel laureate David Baltimore, president of the California Institute of Technology in Pasadena. It recommended that individual universities and research institutions set up committees to comply with biosecurity regulations and promote self-governance.

Experts at the Geneva meeting say that the conference is unlikely to lead to a binding international agreement on codes of conduct. But they hope it will encourage individuals and institutions to take action.

Erika Check

"Many biologists still do not believe that their work could possibly be misused."



THE COOLING EFFECTS OF VOLCANOES

Sulphur emissions stunt the growth of methane-releasing bacteria.

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PUNCHSTOCK

US teams join hands to build dexterous robots

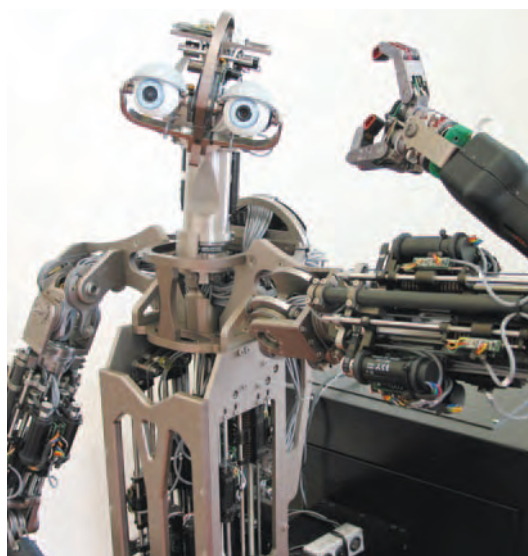
CAMBRIDGE, MASSACHUSETTS

After years of following increasingly isolated paths, robotics researchers in the United States have agreed on a common goal: making machines that are good with their hands. They hope that a unified scientific front will help them to compete against groups in Asia, where research into humanoid robots has been heavily funded while cash for US projects has dwindled.

At the first annual 'Robotics: science and systems' conference, held at the Massachusetts Institute of Technology (MIT) in Cambridge last week, delegates embraced the idea of pooling their resources.

"A bunch of people realized what we're all doing fits together — this is a field," says Oliver Brock, co-director of the Laboratory for Perceptual Robotics at the University of Massachusetts, Amherst. "We're bringing together the engineering and science to automate the performance of physical work."

And in March, a group of robotics experts meeting in Houston, Texas, agreed that US researchers should work together to develop robots that can move around and do useful work: a field called autonomous mobile manipulation (AMM). This made more sense than trying to compete individually with the strengths of foreign groups, such as the two-legged walking robots being developed in Japan and Korea, they decided. A four-year,



Handy device: Domo is able to track and grasp balls.

\$14-million NASA initiative to support AMM was launched this spring.

AMM could revolutionize areas such as manufacturing, agriculture and space applications, adds Rodney Brooks, director of MIT's Computer Science and Artificial Intelligence Laboratory. "There's plenty of reason to believe that robot manipulation will change the world."

To make his point, he unveiled one of the most advanced projects at the conference: a robot called Domo. Built by PhD student Aaron

Edsinger-Gonzales and research engineer Jeff Weber, Domo combines computer vision, force-controlled movements and tactile sensing. So far, it can track the motion of a coloured ball and reach out with one or both hands to grasp it. Brooks hopes that it will learn to feel its way around and be able to manipulate objects with the dexterity of a six-year-old child.

But not everyone is happy. One reason for building robots that can do specific tasks, such as using hand tools, is to learn more about the underlying principles of those actions in humans. Some researchers worry that the AMM approach will favour technological advances at the expense of basic research. "There's no science yet," one MIT researcher not involved in the project says of Domo. "But it's a sweet robot."

The fear of falling behind foreign competitors is likely to keep US researchers working towards a common technological goal. Companies such as Toyota, Honda and Sony all have major programmes to develop humanoid robots, for example, and Japan reportedly plans to invest billions of dollars in the field over the next five years.

"They'll have all the patents," says Alan Peters, a robotics expert at Vanderbilt University in Nashville, Tennessee. "It will be as if the transistor were invented in Japan, and Bill Gates was named Kobayashi."

Gregory Huang

A. EDSINGER-GONZALES

Whaling divisions deepen as Japan pushes for credibility

TOKYO

The annual meeting of the International Whaling Commission (IWC) this month is set to take a different tone from previous gatherings. For the first time, the pro-whaling lobby seems to have amassed sufficient numbers to exercise a majority.

The IWC has long struggled to balance the competing demands of its remit to conserve whale stocks and develop a sustainable whaling industry. Its membership has been bitterly divided since 1986, when the commission introduced a moratorium on commercial whaling.

The pro-whaling block, led by Japan, has rarely got its way. The commission acts more like a conservation organization than one geared towards regulating commercial whaling, complains Joji Morishita of Japan's

fishery agency. He says that, with a majority, the pro-whaling nations would steer the IWC back towards its original purpose.

The pro-whaling lobby does not yet have enough members to quash the moratorium on commercial whaling, which would require a three-quarters majority. But it may be able to push the IWC to axe several subcommittees, such as those devoted to conservation or the impact of whaling on whale-watching, says Phillip Clapham, a marine biologist at the National Marine Mammal Laboratory in Seattle.

It could also help Japan to score some much needed political credibility points. Since the moratorium, Japan has killed more than 8,000 whales for scientific research. This is permitted under IWC rules, although Japan's programme has

never been approved by the commission. At the IWC's annual meeting in Ulsan, South Korea, on 20–24 June, Japan is expected to table a proposal that would double its annual take for research purposes.

But anti-whaling nations believe the programmes have little scientific benefit. Although Japan doesn't need IWC backing to carry out scientific whaling, formal approval for either its previous programme or its latest proposals would bolster the country's image (see pages 856 and 883).

Morishita says that the extra catch will provide data that anti-whalers say are needed before commercial whaling can resume. "As it gets more political, the scientists come up with more difficult questions, and these require more data," he says.

David Cyranoski

SPECIAL REPORT

Gloomy outlook for Blair

British prime minister Tony Blair hopes to make significant progress on climate change at the upcoming G8 summit. The United States is standing in his way, but his efforts may at least benefit climate research.

You could forgive Tony Blair for wishing he had never made climate change a priority for the G8 summit in July. The British prime minister's proposals for emissions targets got a frosty reception when he put them to US President George W. Bush last week. And mounting claims that the Bush administration manipulates climate science suggest that the United States is still some way from agreeing to concrete action (see 'Increasing the uncertainty', below).

But Blair's efforts have not been a complete washout. There is still talk of significant investment in climate-related research and technology. And Britain has a plan on the table to address one major scientific concern: Africa's lack of input to the global network of weather stations (see 'Solving Africa's climate-data problem', right).

Blair initially had three broad climate-related aims for the meeting of the Group of Eight industrialized nations, to be held in Gleneagles, Scotland, over 6–8 July. He planned to rely on science to set emissions targets; to agree on new technologies that could help to achieve that goal; and to build a climate consensus with the world's emerging economies.

Such lofty goals were always going to be a long shot, and a document leaked late last



The heat is on: Tony Blair and George W. Bush do not see eye to eye on climate change.

month seemed to confirm what many had believed to be the most likely outcome — there was as yet no agreement on setting stricter emissions targets.

The world's leading science academies were moved to lend their voices to the cause. The academies of all the G8 countries, along with those of China, Brazil and India, issued a strongly worded statement on 7 June calling for immediate steps to reduce greenhouse-gas emissions. "The scientific understanding of

climate change is now sufficiently clear to justify nations taking prompt action," they declared.

Yet even a shared statement could not blur the sharp divide between the US and UK positions. The president of Britain's Royal Society, Robert May, criticized US climate policy as "misguided". And Blair's attempts to persuade Bush of the need to act were not rewarded.

After a meeting in Washington on 7 June, Bush reiterated his position that more research is needed before action can be taken on climate change. He did not budge on the issue of emissions targets; the United States remains the only G8 country that has not signed the Kyoto Protocol on climate change.

Blair has now turned his attention to leaders in Europe and elsewhere, starting this week by enlisting Russian president Vladimir Putin in the fight against climate change. But it is true, as ever, that without the United States, any emissions agreement will be weak at best.

Instead, the outcome of the summit seems most likely to be increased investment in sustainable and renewable technologies. The leaked statement, which purports to be an early draft of a G8 climate agreement, suggests spending money on relatively small fixes, such as improving the energy efficiency of buildings, encouraging the development of hydrogen and other fuel-efficient cars, and developing better methods to capture and store carbon dioxide emissions. It recommends an international 'carbon challenge' prize to stimulate such research. ■

Increasing the uncertainty

Fresh accusations that the Bush administration manipulates climate science for political ends could spell trouble during the G8 meeting.

Philip Cooney, a political adviser to President George W. Bush and a former oil-industry lobbyist, made hundreds of changes to a summary of research conducted under the Climate Change Science Program, the Bush administration's main climate research initiative. As the report was being finalized in 2002, Cooney added phrases such as "significant uncertainty" to describe the current state of scientific knowledge, and cut passages that were inconsistent with White House policies.

Administration officials say the changes were simply part of a routine vetting process. "The reviews were pretty standard," says John Marburger, Bush's science adviser. But critics charge that the net effect was to exaggerate the level of uncertainty about the evidence for climate change. The changes became public

after the Government Accountability Project, a Washington-based watchdog group, supplied copies of Cooney's edits to *The New York Times*.

Cooney resigned last week from his post as chief of staff for the White House's Council on Environmental Quality. White House officials say he left for reasons unrelated to the edits.

Eileen Claussen, president of the Pew Center on Global Climate Change, an environmental think-tank in Arlington, Virginia, says the editing fiasco makes the Bush administration look insincere when it talks about the uncertainties of climate change. "This shows that the United States is isolated from the rest of the world," she says. Congressman Henry Waxman (Democrat, California) and Senator John Kerry (Democrat, Massachusetts) have called for an investigation into changes made by White House officials to climate-change documents produced by scientists.

Geoff Brumfiel, Washington DC

D. BRACK/PHOTOGRAPHER SHOWCASE/NEWS.COM

Solving Africa's climate-data problem

World leaders are poised to confront one of climate science's biggest problems — a gaping hole in global climate data left by Africa's dilapidated weather stations.

Nature has learned of a British proposal in which a relatively small investment in African data could dramatically improve models of global climate change and its impact on the continent. The plan will be discussed at next month's G8 summit.

But the proposal must tiptoe around some sensitive political concerns. Observers say that a few countries may be withholding weather data in order to sell it to Western research organizations — a charge strongly denied by African meteorological agencies.

Decades ago, Africa had a relatively dense network of stations to measure rainfall, temperature and other weather data. But some areas, such as the Sahara, have always been sparsely covered, and some weather centres have aged badly as cash-strapped governments have been reluctant to invest in trained staff and equipment.

Many of the stations now sit silent (see map), and their density in Africa is eight times lower than the minimum recommended by the World Meteorological Organization (WMO). "This acts as a huge brake on climate science," says Richard Washington, a specialist on African climate at the University of Oxford, UK.

Africa suffers because weather data can help local health planners fight malaria, which is strongly influenced by periods of drought or heavy rainfall. The lack of data also hampers climate researchers around the globe, who need historical data and input from current events to improve their models.

Western meteorologists who have worked in Africa say some centres there fear that releasing data might allow outside companies

to generate forecasts for agriculture, aviation and other industries — a function currently performed by state-run agencies. "There is a degree of protectiveness," says Mike Harrison of the UK Met Office in Exeter.

Others argue that historical records are viewed as bargaining chips. "Some conclude that there is money to be made," says Richard Thigpen, a WMO official who works on access to African weather data.

African meteorological services contacted by *Nature* denied that political or financial pressures affect the data flow. Tony Anuforom of the Nigerian Meteorological Agency in Abuja accepts that data from his country's weather stations is not relayed promptly to the WMO. But he says the agency is installing a satellite link that should remedy the problem.

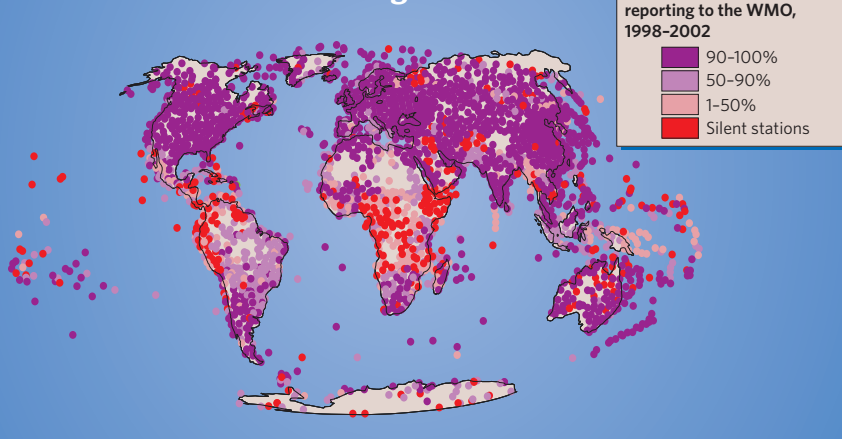
Some outside observers agree. The lack of data "is not a bargaining tool", says Steve Palmer of the UK Met Office. "It's because they can't make the data available." And some measurements, such as those from balloons, are often too expensive.

Meteorologists say the problem can be solved by wealthier nations getting involved. A US\$70-million investment over five years from rich countries could make "significant in-roads" into the problem, says an official at the UK Department for International Development. And there is no need for any new bureaucracy: a draft of a purported G8 climate-change agreement, leaked last month, said the money could be channelled through an existing WMO scheme, the Global Climate Observing System.

But the stations will need money from African governments if they are to survive. And in nations where the education and health infrastructures require major investment, that may be a lot to ask.

Jim Giles, London

Weather stations around the globe



SOURCE: WMO/UK DEPT FOR INTERNATIONAL DEVELOPMENT

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NASA

Geologists call time on dating dispute

LONDON

After more than 150 years of conflict, geologists have taken a small step towards agreeing what to call the time we live in.

The fight concerns time rather than space, yet feelings are running as high as in any border dispute. Supporters of one geological term have been accused of "expansionist tendencies" in their bid to annex neighbouring territory. They, on the other hand, say that their rivals are clinging to meaningless traditions of the past.

As the allegations fly, representatives of both sides voted on the issue last week in the first move towards a solution. But agreeing the details is likely to take months, if not years.

Since at least the nineteenth century, different groups of geologists have used conflicting terms to describe the recent past. Some simply describe the past 23 million years as the Neogene period. But others invoke an extra period — the Quaternary — which follows the Neogene and began around 2 million years ago.

The row has recently escalated, as a result of a seven-year international project that was meant to resolve such arguments. This involved finding and dating deposits around the world that mark transitions between periods, such as the appearance of a new type of rock, or the emergence of a new species in the local fossil record. The results were published last year, along with a definitive geological timescale (see *Nature* **429**, 124–125; 2004).

But as the scale was being developed, Quaternary researchers learnt that their period had disappeared — absorbed by the Neogene. The result was a lot of angry geologists. "They tried to do away with it without anyone noticing," says Philip Gibbard, a Quaternary researcher at the University of Cambridge, UK. "I get an e-mail a day complaining about it."

Gibbard and his colleagues have since campaigned hard for the period to be restored. They say that there are clear geological records, such as evidence for an increase in the number of icebergs and glaciers, that mark the beginning of the Quaternary, and that the period has a unique identity, characterized by periods of glaciation and the emergence of humans.

The campaigners say that the fight is about more than just a name: if the term is removed from the time scale, they fear that their field

Period piece: glaciers are cited by some researchers as key evidence for a division in geological time.

could lose its identity and be deprived of the status it is due. "Thousands of scientists identify with this period," says Gibbard. "They do not take lightly to being labelled Neogene researchers." The term is certainly embedded in the research community; it appears in the name of at least seven societies and four journals around the world.

Those behind the latest timescale admit that the process could have been handled better. "I understand why they got angry," says Felix Gradstein of the University of Oslo, lead author of the timescale. "I apologize."

But behind his conciliatory tone lies an insistence that the Quaternary researchers do not have enough evidence to claim ownership of a separate period.

Gradstein and others argue that cooling events occurred several times during the past 60 million years and so are not suitable markers for a new period. They also point to the Quaternary community's long-standing disagreement over exactly when the period

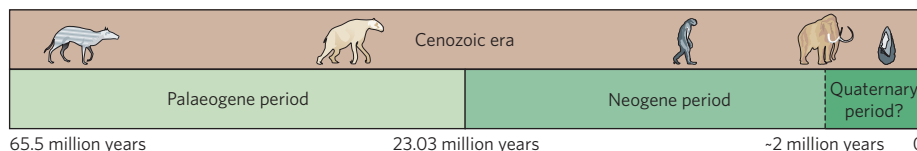
begins, and the fact that the Quaternary is a hangover from a previous naming system, the rest of which has been discarded.

And they seem to have had enough when it comes to allegations of expansionism. "I have seen a lot of nonsense from some hot-tempered colleagues," says Gradstein.

But a resolution might finally be in sight. An eight-member task force, set up last August by the International Commission on Stratigraphy (ICS), the organization that coordinated the timescale, and the International Union for Quaternary Research, voted last week to retain the name Quaternary in some form, and to define its onset as 2.6 million years ago.

It is now that the hard work really begins, says panel secretary James Ogg of Purdue University in West Lafayette, Indiana. Task-force members have to decide how to rank the Quaternary and will present their results to an ICS meeting this September in Leuven, Belgium. Should it remain a period or be downgraded to a subperiod? Or perhaps be removed officially from the hierarchy of the time series, but retained as an informal unit? "None of these will make everyone happy," warns Ogg. "Of course, the issue could be left undecided for another decade..."

Jim Giles



F. TODD/BRYAN & CHERRY ALEXANDER PHOTOGRAPHY

ON THE RECORD

“Cloning a human being is nonsense. Briefly, it is not ethical, it is not safe at all, and it’s technically impossible.”

South Korean stem-cell pioneer Woo Suk Hwang responds to fears about human cloning.

“We won’t be asking passengers to leave a sample and we won’t be carrying a resident sheep at the back of the bus.”

Andrew Dyer, managing director of Stagecoach South, UK, explains plans to use sheep urine to reduce exhaust emissions on its buses.

SCORECARD

**Creative space**

The European Space Agency is drawing up

plans to put art into orbit, and is canvassing ideas on how to bring high culture to the Space Station. The results could truly be out of this world.

**Rub a chicken**

At last — it’s possible to pet a hen online. Stroking

a model chicken sends signals winging across the Internet to a bird wearing a special jacket. The coat lets the hen feel the caresses as if they were real. It’s enough to cure any fowl mood.

**In-flight chitchat**

Plans to allow mobile phone calls on planes

have riled radioastronomers, who fear that the inane chatter will drown out the faint signals from the stars.

DATAPOINT

Percentage of Americans who rate the quality of the environment as ‘good’ or ‘excellent’.

Democrats 27%

Environmentalists 34%

SUV owners 48%

Source: Survey on American Attitudes on the Environment (Yale Univ. School of Forestry & Environmental Studies, 2005).

German tobacco papers reveal lump sums for health experts

MUNICH

The tobacco industry is now notorious for its attempts to influence the public-health sector in recent decades. Now an analysis of millions of industry documents reveals just how successful companies were at enlisting Germany’s top health experts in tobacco-friendly studies — a tactic that many believe is responsible for the country’s lax antismoking laws today.

International firms such as Philip Morris, R. J. Reynolds and Brown & Williamson paid millions of deutschmarks in the 1980s and 1990s to leading health experts such as Karl Überla, who was then president of Germany’s federal public-health department, and Jürgen von Troschke, head of the German Coordinating Agency for Public Health, based at Freiburg University. The allegations were published last week in the news magazine *Der Spiegel*.

The study was commissioned by the German Cancer Research Center (DKFZ) in Heidelberg, which asked sociologist Dietmar Jazbinsek to search Internet archives of more than 40 million documents that a US court forced the tobacco industry to make public in 1998. He was continuing work started by the US physician Norbert Hirschhorn, who published the results of a scan of 600 documents in 2000 (*N. Hirschhorn Tobacco Control* 9, 242–248; 2000).

Jazbinsek identified the country’s 15 most prominent public-health experts and searched the tobacco archives using various spellings of their names. He discovered that at least four of the 15 had received large sums from industry, mostly through research bodies, to produce reports on the risks and benefits of smoking.

Martina Pötschke-Langer, head of the cancer prevention unit at the DKFZ, says she is stunned by the size of the scandal. “It seems as if hundreds of German researchers and scientific societies have been involved,” she says.

The biggest sum went to Überla, who got DM1.6 million (US\$1 million) in 1982 from a German tobacco industry association to do a study into the risk of passive smoking. The work, published in the early 1990s in the non-peer-reviewed, German-language journal *Münchener Medizinische Wochenschrift*, played down the risk, according to cancer researchers.

Von Troschke received DM40,000 in the

same year for a literature review about smoker motivation. In subsequent talks and publications he frequently referred to the “psychosocial benefits of smoking”. In a popular book in 1987, von Troschke warned of discrimination against smokers. He described a “blood-thirsty mood” in the German public, which he compared to the Nazi’s persecution of Jews.

The other two experts named in the report are Johannes Siegrist, director of the Institute of Medical Sociology at the University of Düsseldorf, and Johannes Gostomzyk, a researcher at the Augsburg health authority. Siegrist admitted to *Nature* that he received three payments, but he says that his studies were independent and that he feels deceived by the way the industry used his results. Überla and von Troschke admitted to *Der Spiegel* that payments were

made, although both insist that the funding did not bias their studies. Neither they nor Gostomzyk responded to *Nature*’s requests for an interview.

There is no suggestion that the four experts made up or manipulated scientific data.

Most of their studies could be classified as ‘soft’ science, relying on the presentation of different arguments, says Jazbinsek. But he says they all tended towards views favoured by industry. “It is absolutely mad that top public-health experts — of all people — accept funds from the tobacco industry,” Jazbinsek says. “These collaborations have been good for their careers.”

Many, including Pötschke-Langer, believe that the stance of those such as Überla is part of the reason that effective non-smoking legislation was not implemented in Germany until recently. Although the country has ratified the World Health Organization’s Framework Convention on Tobacco Control, which came into force in February, its antismoking legislation lags far behind that of the United States, Canada and many European countries.

Ironically, the DKFZ itself accepted grants from the tobacco industry, until a new director ended the practice in 1982. Some payments to scientists continue, such as the Philip Morris research prize, for example. But scientists at the DKFZ are urgently calling for an ethical code that will oblige scientists and health researchers — and creative artists — to reject all sponsorship from the tobacco industry. ■

Quirin Schiermeier

**MAYA WAY**

The arrangement of corpses in a tomb suggests women held a strong position in Mayan culture.

www.nature.com/news

WAKÁ RESEARCH TEAM

Look out for rough drafts of mammal genomes

WASHINGTON DC

The scaly pangolin, the wide-eyed bushbaby and a deadly mosquito are among 13 new organisms to be sequenced by the US National Human Genome Research Institute (NHGRI) in Bethesda, Maryland.

But not all of their genomes will be read completely. The agency, which has 61 genomes finished or in the pipeline, said on 7 June that it will fund a strategy called low-density draft coverage for eight of the mammals in the new batch, including the pangolin (*Manis* spp.) and bushbaby (*Otolemur garnettii*). The approach has been used on six animals already, including a hedgehog-like animal known as a tenrec (*Echinops telfairi*). The process can reveal mammals' evolution by providing information from distantly related species.

Low-density coverage will help biologists to find the genome regions that have changed least through time. But many

IMAGE
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The bushbaby's DNA sequence may be a bit skimpy.

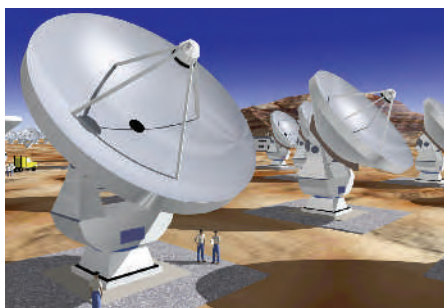
scientists are disappointed because it may not address one of the hot areas of genomics: large differences in genomes, such as rearrangements of DNA or changes

in the number of copies of particular regions of DNA (see *Nature* 435, 252–253; 2005). Finding such regions is likely to require high-quality, complete genome assemblies. To address this, the NHGRI says it will fund a project to sequence part of the gibbon genome, which seems to contain lots of genomic rearrangements.

But genome biologists are pleased, at least, that new species are up for sequencing. They accept that, in many cases, low-density coverage is the best they will get for the time being. "At today's cost and effort we're not going to see a finished genome for the tenrec," says Richard Gibbs, director of the Human Genome Sequencing Center at Baylor College of Medicine in Houston, Texas. But he says he hopes to find out more about the tenrec and its relatives as sequencing costs drop. "This is a beginning, not an end, to exploring those other species." ■
Erika Check

A. BANNISTER/GALLO IMAGES/CORBIS

ESO



False economy? Making the Atacama Large Millimeter Array smaller would harm its science.

US academy counts cost of shrinking radio telescope

One of the world's largest radio telescopes, being built in Chile, may be cut back so much that its scientific importance will be limited.

Originally expected to cost US\$650 million, the Atacama Large Millimeter Array (ALMA) is now overrunning its budget, prompting officials to consider cutting the number of planned operational antennas from 60 to 50 or even 40. A report issued on 10 June by the US National Academy of Sciences, says that either change would prevent the project from producing revolutionary science, although it still might yield "transformational results".

The antennas will detect millimetre- and submillimetre-length electromagnetic emissions from embryonic stars, planets and galaxies.

ALMA is a project of the US National Radio Astronomy Observatory, the European Southern Observatory and Chile. In April, the telescope's governing board decided that a minimum of 50 antennas would be acceptable. A final decision on the number of antennas could come by the end of the year.

Abstentions scupper vote on Italian fertility laws

Italian biologists were disappointed on 13 June by the failure of a referendum that would have relaxed the country's strict fertility laws.

Barely half the 50% of voters needed to reach a quorum turned out, in part because the Vatican called for a boycott of the poll. Among other things, the proposal would have legalized preimplantation genetic diagnosis and research on human embryonic stem cells.

Scientists campaigning for a 'yes' vote complained that the 'no' camp misinformed the public about the issues and acted undemocratically in urging abstention.

The scale of the defeat means that there is unlikely to be another chance to liberalize the laws in the near future.

Rocky planet is smallest seen beyond Solar System

Astronomers have found the smallest planet yet outside our Solar System, weighing in at just seven-and-a-half times the mass of Earth. Its discoverers say that the planet is likely to be rocky, rather than a gas giant like Jupiter.

"This is the smallest extrasolar planet yet detected and the first of a new class of rocky terrestrial planets," says Paul Butler of the Carnegie Institution in Washington, part of the team that found the planet. "It's like Earth's bigger cousin."

The planet orbits the star Gliese 876 once every two days. The star, a red dwarf that lies 15 light years from our own Solar System, is already known to have two Jupiter-sized planets.

The planet's surface temperature probably exceeds 200 °C, the astronomers say, because the planet orbits just 3 million kilometres from the star, more than ten times closer than Mercury's orbit is to the Sun.

Study calls for controls on marine bioprospecting

An international system is needed to protect the oceans from damage by bioprospecting, says a new study from the United Nations University in Yokohama, Japan.

The report, issued on 8 June in Tokyo, is intended to influence a UN workshop next March on expeditions in international waters. The report's authors worry that

some nations could unfairly exploit deep-sea resources.

The United States, Japan and France are expected to be the strongest opponents of limits on exploration. The demand for new drugs and industrial materials has recently targeted marine organisms, particularly extremophiles that live in high-pressure, high-temperature environments (see *Nature* 429, 598; 2004).

▶ www.ias.unu.edu/binaries2/DeepSeabed_final.pdf

Libyan police cleared of torturing Bulgarian nurses

A Libyan court last week cleared nine policemen of torturing a group of foreign medical workers in a Tripoli jail.

The five Bulgarian nurses and a Palestinian doctor were sentenced to death in May 2004 after being convicted of deliberately infecting hundreds of children with HIV. But the six say that their confessions were extracted under torture.

The policemen's acquittal on the torture charges is "a matter for serious concern", says Benita Ferrero-Waldner, the European Union's commissioner for external relations. She says that during a recent visit to Libya, authorities assured her that due process of law would be upheld.

"We have been extremely disappointed by the procedures in this trial," she says. Libya's supreme court will rule on the medics' fate in November. Given last week's verdict, Ferrero-Waldner says, it is essential that the court should "bring this issue to an appropriate and humanitarian conclusion".

Australia plans 'test-tube' sharks to aid ailing species



Life is tough for the grey nurse shark (*Carcharias taurus*). From the moment it hatches inside its mother's uterus, a baby grey nurse has to avoid being devoured by its siblings. Only one embryo will survive the intrauterine cannibalism — and this survivor faces a bleak future.

Hunting has slashed shark populations to critical levels. For decades, the fearsome-looking grey nurse shark was targeted as a possible man-eater, although it prefers fish to surfers.

Now Australian scientists hope to bolster shark numbers by growing embryos in fake uteruses. A team at New South Wales's Department of Primary Industries plans to remove fertilized eggs from wild females before the embryos start eating each other. The eggs will be reared in artificial humidicribs until the pups are old enough to fend for themselves.

Conservation groups say that instead of hatching 'test tube' sharks, more effort should be spent on protecting the sharks' natural habitat and breeding grounds.

NSW DEPT. PRIMARY INDUSTRIES

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UNDER THE GUN

Western conservation biologists working in Myanmar have been accused of colluding with a brutal military regime — charges they deny. **Duncan Graham-Rowe** reports from this pariah state.

In March 1997, Chris Wemmer, a biologist with the Smithsonian National Zoological Park in Washington DC, received an alarming fax. It was an article from the British *Observer* newspaper accusing him of colluding with the Burmese junta in committing human rights abuses in the country now known as Myanmar.

The article, headlined “Save the rhino, kill the people”, implicated Wemmer’s organization in the murder and forced removal of ethnic Karen people to make way for a huge wildlife park, called the Myinmoletkhat Reserve. It criticized the Smithsonian Institution for being one of the first Western organizations to work with the regime “since it massacred 3,000 demonstrators in 1988”.

Wemmer still fumes about the article, which he claims misrepresented the Smithsonian’s involvement in this secretive southeast Asian nation. That the institution’s project, in a wildlife park called Chatthin, headed by a

Karen warden, was based 1,200 kilometres north of the site of the atrocities described in the piece didn’t seem to matter, he complains: “We were guilty by association.”

The next day, Wemmer was summoned to Capitol Hill, to answer congressional staffers’ questions about his involvement with the Burmese regime, then called the State Law and Order Restoration Council. They wanted to know why a federally funded institution was operating in a country against which the United States enforced sanctions. They eventually accepted Wemmer’s arguments that his work was benign. But the biologist left the meeting knowing that he was on thin ice. “We were working in a highly charged political arena, and stood accused of hurting Myanmar’s democratic movement,” Wemmer says.

Similar accusations still swirl around Wemmer and other Western conservation biologists who work in Myanmar. The researchers are convinced that their work is justified, given

the country’s rich yet threatened biodiversity — and the enthusiasm of local conservationists for partnerships with Western scientists. But critics worry that their presence helps to legitimize the military regime, and seems to prioritize the needs of wildlife above those of a brutally repressed population.

Innocents abroad?

When he first visited Myanmar in April 1988, Wemmer did not know what he was getting into. “I didn’t do a great deal of soul searching before going out there,” he admits. His first impressions were simply of a very poor and isolated country. “That was about all I knew,” says Wemmer. “I think most field biologists are not particularly well informed about the countries in which they work.”

But four months later, Myanmar erupted into the headlines when a mass demonstration in the capital, Yangon (formerly Rangoon), ended with a massacre often called ‘Burma’s

Tiananmen Square'. Since then, Myanmar's military rulers, who now call themselves the State Peace and Development Council, have been heaped with opprobrium. They are at war with the Karens and other ethnic minorities, and rule the Burmese with a rod of iron. The London-based group Burma Campaign, which is pressing for democracy in the country, maintains that adults and children are routinely coerced into working on government projects; more than a million people have been relocated to make way for military installations, business ventures and the wildlife park highlighted in the *Observer* article. And according to Amnesty International, "torture has become an institution". Meanwhile, Nobel peace laureate Aung San Suu Kyi is under house arrest, 17 years after she was elected to lead the country.

Asset stripping

Wemmer and his colleagues agree the regime is unsavoury. But they reject the accusation of collusion in human-rights abuse. "If I thought what I was doing out there was aiding the regime in abusing the people, my conscience wouldn't allow me to stay. I would get the hell out," says Wemmer. "But by talking to a lot of poor people in the country and getting a really intimate understanding of how they feel, I came to the conclusion that what we are doing is basically a positive thing. Not being there wouldn't help the cause in any way."

For conservationists, the main motivation is the opportunity to protect one of Asia's last wildernesses. "Myanmar has the largest area of standing forest in the whole of the Asian-Pacific region," says Alan Rabinowitz, director of science and exploration for the Wildlife Conservation Society, based at the Bronx Zoo in New York, who has worked in the country for many years. The forests are a biodiversity hotspot and a stronghold for endangered tigers and Asian elephants.

But maybe not for much longer: logging is rife, with much of the profits believed to go to Myanmar's military rulers. According to the London-based pressure group Global Witness, which works to highlight the links between the pillage of natural resources and abuses of human rights, Myanmar exported more than 1.72 million cubic metres of timber between 1999 and 2000.

If this trend continues, the country will fast go the way of neighbouring Thailand, which has lost more than half of its



forests over the past 30 years. For Jonathan Eames, who works for the Indochina arm of BirdLife International, the threat to Myanmar's biodiversity demands immediate action. "If we wait five or ten years in the hope that there's going to be a transition to a democratic, freer Myanmar, it will be too late," says Eames, who is now working on a US\$1-million project to set up a reserve at Natmataung in the south of the country.

But are biologists such as Eames, Rabinowitz and Wemmer letting their passion for conservation override concerns about legitimizing a brutal regime? To assess the situation, I travelled in May to Hukaung, in the

northern state of Kachin, where the Wildlife Conservation Society is involved in a project to establish the world's largest tiger reserve, covering some 22,000 square kilometres. The park has around 100 tigers, and the potential to support ten times that number.

Under surveillance

Arriving in Myanmar, the first impression is of its friendly, hospitable people. But political reality soon creeps in. Soldiers scan your documents at regular checkpoints, while locals glance anxiously over their shoulders before starting a conversation. Propaganda posters express the "People's Desire" to crush troublemakers and oppose interfering foreigners. And in Tenai, an ugly mining town that hosts the Hukaung tiger reserve's headquarters, a man from military intelligence asked my guide daily questions about my movements.

But in the park, there's little official interference, and few reminders that you're in a country reviled for its human-rights record. During my visit I accompanied three rangers on a trek into the

jungle to set camera 'traps' used to count tigers. There are 20 rangers, mainly young men who spend 21 days each month in the field, unarmed and charged with protecting an area the size of Vermont against poachers, illegal gold miners and potentially hostile nomadic hunters. Part of their job is to teach local people about the benefits of conservation and discourage them from hunting.

It is tough work. On our way to the field sites, we were plagued by leeches, slashed by razor-sharp, face-high grasses, and had to force our way through bamboo thickets so dense that we could barely swing our bush knives. Convincing the impoverished people

of this region that it is better to conserve tigers than to hunt them for the lucrative Chinese medicine market is very difficult.

It is clear that the park rangers, and Burmese conservationists, appreciate the presence of Western biologists. Myanmar needs foreign expertise, money and equipment, says U Gar, a retired forestry official who helped set up the Biodiversity and Nature Conservation Association, the country's first environmental group. "We can't wait, because our natural resources are depleting at an alarming rate," he argues. "Doing something is better than doing nothing."

Western involvement may also help keep the authorities honest, when it comes to

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Timber land: Myanmar's huge forests are being logged rapidly for export.

conservation. Too often, protected reserves are merely 'paper parks'. One of the most egregious examples is the Pedaung Wildlife Reserve, a small park near Myitkyina, the capital of Kachin, which I passed through on my way to Hukaung. It is Myanmar's oldest reserve, but arguably the most degraded.

The warden, Khin Maung Hla, told me that since the park was established in 1918 it has suffered nearly 40% deforestation in its lowlands. The rhino, elephants and tigers that once roamed the forests are long gone. Even after the government gave Pedaung a new charter for wildlife protection in 1992, the degradation accelerated: three army compounds, a railway and a computing college have been built in the park.

Efforts at reforestation by the warden and local people consist of plantations of mangoes, lychees and other fruit trees. Far from restoring the area's natural biodiversity, these are monocultures planted to feed soldiers and make money.

Uneasy alliance

Rabinowitz vows that this won't be repeated at the Hukaung tiger reserve. "But it is a huge challenge," he says. I counted seven government-backed gold mines on a map of the park; no one knows how many illegal miners are in the area, eroding riverbanks and polluting the water with mercury. But the illicit miners, mostly migrants from China, aren't making huge profits. So Rabinowitz is confident that — with the Wildlife Conservation Society's continued involvement — they will eventually be moved on, allowing the park to concentrate on protecting tigers and other wildlife.

There is plenty to protect: during my trek we saw tracks of wild Asian elephants, the endangered fishing cat and a variety of endemic deer. I even learned of a recent unconfirmed sighting of the critically endangered pink-headed duck — on a hunter's skewer.

The Hukaung reserve also represents an uneasy collaboration between the Burmese government and one of its ethnic opponents, the Kachin Independence Army (KIA). As part of a ceasefire deal, 80% of the park is under KIA control. Rabinowitz has found it uncomfortable wrestling with Myanmar's ethnic tensions, but he is happy with the end result. "I have been playing the middle man, which is incredibly difficult," he says. "I'm being manipulated by both sides, but unless it's to do some evil I don't care because I am manipulating them into saving tigers."

Rabinowitz also argues that Myanmar's record on human rights may not be as despicable as is generally believed — at least when judged against the standards of other countries in the region. "The displaced people from



Middle man: Alan Rabinowitz (centre) has had to work with both government and guerrillas.

Burma are a very intelligent, educated group who have maintained a hugely strong lobby," he says. "I'm not arrogant enough to say I have seen everything there is to see. But having worked in the country for ten years, travelling to the most remote areas, I think it's been blown out of proportion."

Eames also questions whether the abuses in Myanmar are of a fundamentally different

magnitude to those in neighbouring countries, asking: "Where do you draw the line?" Do you stop working in countries such as China, which has countless political prisoners and has annexed Tibet? What about Indonesia,

where militias linked to the government slaughtered one fifth of the population of East Timor before it eventually gained independence in 2002? Even tourist-friendly Thailand last year cracked down on Muslim dissidents, Eames notes.

Activists agree that human rights are being trampled across much of Asia, but argue that Myanmar gives cause for concern. "It is a

military dictatorship where human rights are severely denied," says Sarah Green of Amnesty International, pointing to the country's 1,400 political prisoners. Another issue, highlighted by Human Rights Watch, is the recruitment of child soldiers — some 70,000 members of the Burmese army are believed to be less than 16 years old. And according to the International Labour Organization, forced labour is widespread. The authorities claim this is part of Buddhist tradition in which people donate their labour for the good of the community. But it's difficult to reconcile a practice traditionally associated with the maintenance of temples with the use of 'volunteer' labour to build military barracks, or to clear minefields with scant regard for safety.

Wemmer admits to being troubled by the issue of forced labour, but says that it is hard to pin down exactly what's going on. "You see people working on the roads and highways, mainly women and kids, but there's no chain gang," he says. My experience was similar: there were no obvious signs of oppression and the main struggle, affecting civilians and soldiers alike, seemed to be with rural poverty.

Eames takes a pragmatic view. "I have issues with any country that has political prisoners," he says. "I would prefer it if Myanmar were a liberal democracy, but it isn't."

Game plan

John Jackson, director of the Burma Campaign, doesn't condemn Westerners for working in the country, "provided you can go in and do the work you feel is necessary without buttressing the regime". The problem is that it's impossible to work on conservation projects in Myanmar without engaging on some level with senior officials. Top-down micro-management is so pervasive that it's difficult to get anything done without ministers or generals being involved. And then there is the uncomfortable question of whether the regime is courting Western conservationists to gain international credibility. "I don't think they would do anything without there being a game plan," says Jackson.

This means that conservation biologists working in the country can expect to face continued scrutiny from suspicious politicians. "One has to use a certain amount of charm and guile to persuade these people that what we are doing is not against US interests or aiding and abetting the government of Myanmar," says Wemmer.

When working in an undemocratic country like Myanmar, Wemmer adds, it's important to keep questioning your motives. But in the end, he takes his lead from his Burmese collaborators: "When local conservationists ask for our international support, I don't think it's reasonable to say 'No, we're not going to help you because we don't support your government'."

Duncan Graham-Rowe is a freelance journalist based in Brighton, UK.

"By talking to people and getting an understanding of how they feel, I came to the conclusion that what we are doing is basically a positive thing." — Chris Wemmer

HOTHOUSE HIGH

Do US high schools dedicated to science generate future academics or burnt-out whiz kids? **Kendall Powell** catches up with some of the first pupils to graduate from 'nerd school'.



Sixteen years ago this month John Wilson, Heather Stevens and Rod Rippey graduated with the first class of Thomas Jefferson High School for Science and Technology in Alexandria, Virginia. On a recent Monday evening, in a tavern 15 minutes from their alma mater, they reminisced about their days at the science magnet school.

"I was a super-geek in eighth grade, programming in Basic and Logo on my Apple II Plus," remembers Wilson. "I told my parents, there's this high school where everyone is geeky like me." He pauses, then, "Hey, remember Karel the Robot?"

Stevens groans and explains that Karel was a pseudolanguage for teaching computer programming. "You know, it could only turn left, so to get it to turn right, you had to program in 'turn left, turn left, turn left' — that was when I decided I was not going into computer science," she says. Everyone chuckles — Stevens now works for a software-developing firm in Fairfax, Virginia.

Rippey comes back to the gathering's topic, "Jefferson was designed for me. I didn't fit in at my middle school." But although Wilson and

Rippey jumped at the chance to attend high school with other self-confessed geeks, Stevens went grudgingly at her parents' request. "I had an aptitude in science, but I didn't necessarily like it," she recalls.

It is rocket science

Two decades ago, some of the first science, maths and technology magnet high schools opened in the United States. The Illinois Mathematics and Science Academy (IMSA) in Aurora, outside Chicago, was established the same year as Jefferson in 1985. The magnet concept caught on quickly as a way to challenge the best young minds, and as a possible answer to the decline in US-produced scientists and engineers. There are now 86 science magnet schools nationwide, which select gifted children with an aptitude for science. Australia, Jordan, Israel, Korea, Thailand, Japan and the United Kingdom have set up similar science-focused schools.

But is it a mistake to immerse students in the sciences at the age of 14 or 15? By the time they reach graduate school, such students have already spent eight years in focused study. Is 'nerd' school a place where overachievers

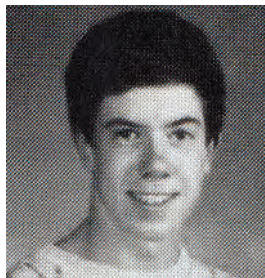
bloom while others wilt under the pressure? Or would their talents be undernourished at a 'normal' high school? Graduates of the high-tech highs give a range of answers.

"It was highly competitive and many thrived on it," says Wilson. "I've never been among so many ridiculous brainiacs since." He, Rippey and Stevens left Jefferson with widely different impressions and goals, but they all ended up in technical careers.

At Jefferson, Wilson soon realized his computer talents paled in comparison with those of others. He shifted to biology and eventually became a psychiatrist working for Fairfax county's mental health department. Rippey's interest in electronics carried him through Jefferson and a college degree in electrical engineering. He has since worked for government military contractors, and at one point was literally a rocket scientist.

"I've always been surrounded by really smart people," he says, high school being just the beginning. But Stevens did not thrive on the competition. Instead, she says, she felt like the dumbest kid in school and describes it as the worst four years of her life.

"School killed my interest in science. I was



Peter Hesse



Matthew Appler



Then and now: Peter Hesse and Matthew Appler flourished at Jefferson High and later founded their own computer-security firms. Today, Jefferson students work together to solve problems in 'circular' discussion groups (right).

TIJHSST TECHNIQUES STAFF



Research is a major part of the final year at Jefferson High.

already burnt-out when I got to college,” she recalls. After college, she rebelled by taking a job at a customs brokerage firm. “I was the smartest person in the company and moved up fast — it was definitely not rocket science,” she says with a nod to Rippey. “I needed that time to feel smart,” she adds. Eventually, her technical abilities led to a career in software.

Go team

The friendship between Jefferson graduates Matt Appler and Peter Hesse is underscored by healthy competition. They took a physics class together in college that turned into a two-man challenge. “And I won,” Appler gloated over burgers recently. “Yeah, but you’re two years older than me,” rebuts Hesse. Each now runs a computer-security company.

Competition inevitably arises when over-achieving students are placed under one roof. “These students are driven from within,” says IMSA principal Eric McLaren. “We knew that we had to foster collaboration and downplay competition.” As such, both Jefferson and the IMSA do not calculate class ranks and have no valedictorian. McLaren says his teachers promote group, rather than individual, efforts.

Team projects and student-led learning methods dominate at magnet schools, where pupils operate far above textbook level. Schooldays tend to be longer and frequently stretch to 12 hours with extracurricular activities and research projects. Schedules run in modules to accommodate in-depth labs and classes lasting up to two hours.

Bright young things

Ande Croll, an IMSA graduate, recalls an interactive physics lesson. Her instructor took the class outside and had them practise throwing spiral passes with an American football. The mechanics lesson on why the spiral improves the pass “sticks with you,” says Croll, a mechanical engineer who now designs electronics for fighter jets in Rockford, Illinois.

This discovery-based learning is everywhere in Jefferson today. In Paul Cammer’s biology class, students form a discussion circle. Cammer has posted an observation about ladybird beetles for the students to explain. He sits at the centre of their circle, nudging plausible ideas forward but rarely intervening. The students lead the debate, and seem unafraid of suggesting crazy ideas or looking stupid.

Cammer keeps them working within reasonable constraints of modern-day research, too. “Remember, you don’t have \$3 billion from the National Science Foundation. You are a high-school student working with beetles in your attic.” The students arrive at a reasonable and testable hypothesis 45 minutes later — in some ways, functioning like they are halfway through graduate school.

“The classes at the IMSA taught you how to think,” says Scott Gaudi, an astronomy post-doctoral fellow at the Harvard-Smithsonian Center for Astrophysics in Cambridge, Massachusetts. “Our classes were: ‘Here’s a problem or something that happened in history, now try to find out why it happened that way,’” he says. “This is the way we do research.”

Gaudi, who searches for planets beyond our Solar System, was named one of the top-20 scientists to watch in the next 20 years by *Discover* magazine. “I would have probably been in astronomy without the IMSA, but I would be nowhere near as successful,” he says. The IMSA and Jefferson both require students to do research projects during their senior year, either in the school labs or with local universities or businesses.

Only a handful of alumni have become aca-

demics, albeit in greater numbers than your average school. But most of them do work in science- or technology-based jobs. In general terms, magnet schools go a long way towards increasing the country’s technical workforce.

“At a time when the United States is producing fewer and fewer scientists, engineers and mathematicians, the IMSA’s doing it. It seems like the school sends more students out every year to do jobs that are technology-based,” says Michael Brody, an IMSA graduate and now a Homeland Security policy adviser for the state of Illinois. The numbers support his claim.

Two-thirds of IMSA graduates earn a degree in science, maths or technology. For females, that is four times the national average. About 40% of alumni earn a graduate degree, with healthcare and computer professions as the top career fields.

Repelled by magnets

But science magnet schools are not for everyone. Chris Colin, a writer and Jefferson graduate, tracked down half the class of 1993 for a book about his classmates. Many of his peers had, like himself, struggled to find careers outside science. “At 14, I didn’t know what the hell I was doing,” he says. Colin says the tunnel vision at Jefferson hindered thinking about other interests and career choices.

Bettie Stegall, a veteran English teacher, uses her creative writing class to push her students to stretch the other side of their brains. The day I visit, her announcement about a poetry assignment receives collective groans.

But some of her students say they wish they had more opportunities to take music, business and other non-science courses. One laments that she had to take summer school twice to fit music into her schedule of required science classes. She plans to major in music at the University of California, Berkeley, in the autumn. “But at another school I probably would have fallen in with the wrong crowd,” she admits. Two-thirds of the seniors say they would not have chosen Jefferson again, but it’s likely that some will change their minds once school is behind them.

Many of the alumni blossomed at Jefferson. Hesse and Appler continue to challenge each other as their firms expand and their toddlers play together. “I would absolutely encourage my kids to go there,” says Appler. “Jefferson brought you out thinking you could do anything.”

Did these alumni also have ‘normal’ high-school experiences? Sure: Brody was elected to the Prom Court, Rippey and Wilson were both on the running team and a senior-year prank involved assembling a Volkswagen Beetle inside the school lounge.

It didn’t hurt to have nerdy football cheers to confuse opponents, either. Stevens chants the final bit over dinner: “Secant-Tangent-Sine-Cosine. Three-point-one-four-one-five-nine!” (That’s pi for the rest of us).

Kendall Powell is a freelance science writer in Broomfield, Colorado.



THISST TECHNIQUES STAFF

KLEIN ET AL. HUM. GENE THER. 2005 (LEFT); ZEPHYR/SPL (CENTRE); A. & H.-F. MICHLER/SPL

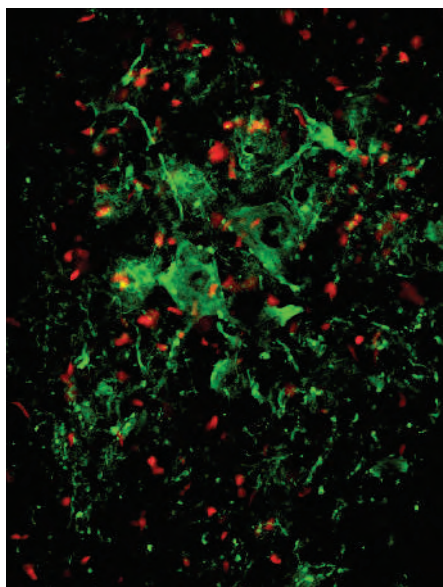


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Ready to use? Stem cells may be able to protect depleted nerve cells (green, left), kill brain tumours (centre, green) or help heal damaged heart muscle.

THE FIRST WAVE

Treatments that use stem cells to replace damaged or diseased tissues are thought to lie many years away. But the cells might find other clinical applications in the near future, says **Catherine Zandonella**.

When patients with the paralysing illness amyotrophic lateral sclerosis (ALS) call Jennifer Brand to ask when stem-cell therapies will be available, she has a stock answer. Brand, who is director of patient services for the California-based ALS Association, says that stem-cell research is still in its infancy. It's just too soon to tell when it might move into clinical trials, she tells her callers.

Indeed, most experts predict that many years of laboratory work will be needed before stem cells can be used reliably to replace damaged cells and tissues. But some enthusiasts argue that this timeline overlooks more immediate clinical opportunities. These researchers want to exploit stem cells' abilities to home in on sites of injury and to deliver biochemicals that protect other cells¹. And, controversially, they hope to move quickly into the clinic. "Disease targets thought to be far in the future are closer to our grasp," claims Evan Snyder, who works at the Burnham Institute in La Jolla, California.

Stem cells have been widely touted as eventual cures for neurodegenerative diseases such as ALS and Parkinson's. The conventional wisdom is that they would be grown to produce the particular nerve cells that are lost in each disease, which would then be grafted into the nervous system to repair it. But researchers currently understand little about the signals

that make stem cells differentiate into particular cell types, nor are they sure how to get grafted cells to integrate effectively into tissues and organs.

Snyder agrees that cell replacement is an exciting future prospect. But apart from replacing lost cells, he notes that stem cells have other, more subtle roles that could be exploited therapeutically. Snyder has evidence that, in the nervous system, stem cells can act as 'chaperones' that nurse sick and injured neurons back to health.

Neural stem cells secrete biochemicals that make the neurons function better, promote survival, decrease inflammation and encourage the growth of blood vessels. One of these factors is glial cell line-derived neurotrophic factor, or GDNF — which seems to protect both the cells that secrete the neurotransmitter dopamine², lost in Parkinson's disease, and the motor neurons that are destroyed in ALS (ref. 3).

Snyder has shown that neural stem cells taken from mouse fetuses secrete GDNF and promote recovery in mouse models of Parkinson's disease⁴. More recently, his team has found that human neural stem cells, from lines originally derived from the brains of aborted fetuses, can migrate from one side of a mouse's brain to the other in response to distress signals issued by injured tissue⁵. The potential to exploit these twin effects therapeutically is clear, Snyder argues. "You are not trying to

replace the lost cells," he says. "Instead, you are trying to protect what is there."

Other researchers are working along similar lines — but are tweaking their cells genetically to make them into better nursemaids. At the University of Wisconsin, Madison, Clive Svendsen's team has engineered fetal neural stem cells so that they pump out greater quantities of GDNF. When the researchers injected these cells into the spinal cords of rats suffering from an ALS-like disease, they survived well and continued to secrete GDNF (ref. 6).

Taking the chance

Svendsen plans to approach the US Food and Drug Administration within the next few months to discuss testing the cells in ALS patients. He believes that the ideal time to give the treatment will be shortly after diagnosis, when a patient begins to lose limb function but before paralysis sets in. "You have a window of about a year-and-a-half to get in and do something," Svendsen argues. Although he hasn't yet published firm evidence from animal experiments that shows his engineered cells are protecting motor neurons, Svendsen has few doubts about pressing ahead into the clinic with a novel experimental therapy — given the severity of the disease and the lack of any effective treatment.

Similar risk-benefit arguments apply for patients with inoperable brain tumours. Here,

too, some researchers are thinking about using genetically engineered stem cells. "The advantage is that these cells can track down and migrate through the tumour," says Frederick Lang, a brain surgeon at the University of Texas M. D. Anderson Cancer Center in Houston. His team has taken cells from bone marrow known as mesenchymal stem cells and inserted a gene for interferon- β — a protein that can kill tumour cells. When the researchers injected the cells into the carotid artery of mice suffering from brain cancer, the cells migrated to the tumour. Encouragingly, these animals lived significantly longer than those who received injections of normal cells⁷.

Heart of the matter

Lang's approach may offer hope for patients who have no other treatment options, but many stem-cell researchers are alarmed about trials for patients with heart disease that are already under way. Based on contested results from animal experiments⁸, clinicians in the United States and Europe are now injecting stem cells into patients' damaged hearts in the hope that they will help repair the damaged tissue.

The problem is that nobody knows for sure whether these cells are differentiating into heart muscle cells, fusing with cells that are already there, or exerting a protective effect by secreting growth factors. It could be a combination of all three, says Emerson Perin, who is heading a study of bone-marrow stem cells injected directly into patients' diseased hearts at the Texas Heart Institute of St Luke's Episcopal Hospital in Houston.

Given the limited understanding of how stem cells behave when injected into the body, some researchers argue that it is too soon to be entering the clinic. For instance, Roger

Barker, a neurologist at the Centre for Brain Repair at the University of Cambridge, UK, worries that neural stem cells might give rise to neurons that could integrate incorrectly into the nervous system, causing adverse effects such as a heightened sensitivity to pain. If so, he fears that the resulting publicity could damage the entire field. "A negative trial doesn't do any good," says Barker.

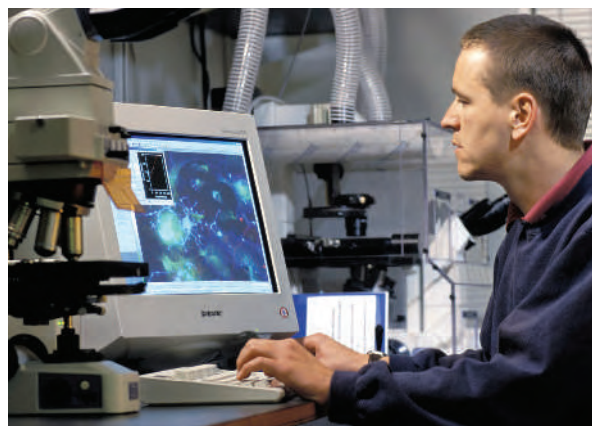
Snyder agrees that caution is necessary, but he argues that early trials using stem cells as nursemaids to protect sick and dying tissues will do the field a service, by giving the regulators and institutional review boards that must approve clinical trials some experience of handling stem-cell protocols. This will blaze a trail for later trials with the loftier goal of replacing damaged tissues, Snyder claims.

In any case, Snyder believes that the risks are relatively constrained for stem cells derived from fetal or adult tissues, provided they are used only in the places where they would normally be found. If cells aren't being put in alien tissues, he argues, they are likely to behave normally.

Growing pains

But embryonic stem cells, which can develop into any of the body's tissues, are another matter. In particular, they can form tumours called teratomas that contain all sorts of tissue types. "You don't want bone or teeth or hair growing inside the spinal cord," says Svendsen. "It just wouldn't look good for the stem-cell field." Nevertheless, some researchers are exploring the idea of using embryonic stem cells to exert nursemaid effects. They note that the cells could be engineered to include a 'suicide' gene that could be activated to kill them, if any problems arise.

Robert Benezra of the Memorial Sloan-Kettering Cancer Center in New York and his colleagues are studying a genetic condition that normally causes female mice to lose their young before birth because of heart defects. When Benezra's team injected pregnant females with mouse embryonic stem cells, they gave birth to live young⁹. The stem cells didn't cross the placenta, but they secreted a heart-repairing substance called



J. MILLER/UNIV. WISCONSIN, MADISON

"You don't want bone or teeth or hair growing inside the spinal cord. It just wouldn't look good for the stem-cell field." — Clive Svendsen

insulin-like growth factor 1, which seemed to protect the fetuses.

Benezra has no plans to move ahead into clinical trials. But cells grown directly from embryonic stem cells could start being injected into patients with paralyzing spinal injuries as early as next year. Hans Keirstead, a stem-cell researcher at the University of California, Irvine, has derived cells that seem to restore some mobility to rats with spinal injuries¹⁰. These cells make the myelin protein coat that serves as electrical insulation for neurons — although Keirstead suspects that other protective mechanisms are also involved. "I believe that they are playing some mysterious 'nurse' role," he says. "They are doing a lot more than just producing myelin."

Keirstead's plan to move rapidly into the clinic has already caused some alarm¹¹. But with other trials of stem cells as nursemaids for sick and dying cells also in the works, patient advocates such as Brand may soon have to revise their message. With luck, these trials will bring fresh hopes for the sufferers of ALS and other debilitating conditions — and not scare stories about adverse reactions.

Catherine Zandonella is a freelance writer in Princeton, New Jersey.

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E. SNYDER

"You are not trying to replace the lost cells. Instead, you are trying to protect what is there." — Evan Snyder

BUSINESS

No longer the upstart

The US biotechnology industry's lobby shop is at last making its mark. Its incoming president may have taken some flak for quitting Congress but, as Meredith Wadman discovers, he's relishing the change.

When the Biotechnology Industry Organization (BIO) held its first annual meeting in Raleigh, North Carolina, in 1993, the new trade group's leaders were thrilled to fill a hotel with 1,400 attendees.

Next week, BIO expects to host 18,000 people at its annual convention in Philadelphia, filling the Pennsylvania Convention Center and 28 nearby hotels with people from 4,600 companies and 55 countries. Melissa Etheridge, the folk-rock singer and cancer sufferer, will perform for the visitors, and golf legend Arnold Palmer, also a cancer survivor, will speak at lunch.

"They have grown from a somewhat-notable to a front-line, high-impact trade organization," says Sheldon Krinsky, a bioethicist and longtime industry observer at Tufts University in Medford, Massachusetts.

"It's a big operation. They have a major presence everywhere," agrees Tony Mazzaschi, an official at the Association of American Medical Colleges in Washington. "In many ways, I think BIO has eclipsed Pharma," he adds, referring to the trade group for the US drug industry.

Jim Greenwood, the former US congressman who in January became BIO's second president, says he is relishing his new position as salesman-in-chief for the industry. "The critical thing is to get people to understand the enormous potential here," he says. "It's mind-boggling, what we're going to be able to do."

Greenwood succeeds Carl Feldbaum, a former prosecutor in the Watergate scandal and Pentagon inspector-general. The ever-accessible Feldbaum oversaw the group's growth from a \$3.8-million operation with 18 staff to today's \$45-million, 104-person lobby shop with offices a ten-minute walk from the White House.

By Washington standards, BIO is still a mid-sized lobbying organization. But it is growing, gaining 225 members in the past year. As well as the small firms that make up most of its



Insider job: Jim Greenwood sees 'mind-boggling' potential in biotechnology.

membership, BIO represents 15 major drug companies, including Pfizer, Merck, Novartis and AstraZeneca, all of which have partnerships with, or stakes in, biotechnology firms. It also contains large agribusiness corporations such as Monsanto, Dow and DuPont.

Fresh direction

When Greenwood was hired last year, he had been leading a congressional investigation into conflict-of-interest policies at the National Institutes of Health, and was just preparing to grill drug-makers on their failure to divulge negative clinical-trial results. He stood down from chairing the hearing, on the grounds that several of the companies being called to testify were BIO members.

His sudden transformation from congressional watchdog to business lobbyist drew some criticism (see *Nature* 430, 495; 2004). But Greenwood says it was a natural progression from his interest in health care. BIO is getting good value from his \$800,000 salary, he says: "It's not a leap up from my predecessor."

Greenwood has made an early mark by hiring other seasoned Washington players. His chief operating officer is Scott Whitaker, former chief-of-staff to then health secretary

Tommy Thompson. His top health-policy adviser, Amit Sachdev, was deputy commissioner for policy at the Food and Drug Administration. "We now have, I think, the strongest health-advocacy team of any healthcare institution in Washington," Greenwood told the Association of Bioscience Financial Officers this month.

At least one of his listeners was impressed. "I like the fact that he came from Congress," says William Baird, chief financial officer at PTC Therapeutics in South Plainfield, New Jersey. "He'll have a level of access that a former staffer or lobbyist will not. After all, these are his former peers."

But some critics accuse BIO of raising false hope of cures for deadly diseases, endangering the planet with genetically modified foods, and producing costly medicines beyond many peoples' reach.

"They've tried to paint themselves as a voice of innovation, a voice of science, a voice of progress. And they've spent a fortune to shield themselves from the very deep concerns that many people have about the real-world practices of the companies behind BIO," says Brian Tokar of the Institute for Social Ecology in Plainfield, Vermont, which is planning a parallel meeting and day of street demonstrations in Philadelphia.

Others less critical of the industry still say it should do more to temper expectations. "I see a lot of the companies still on overdrive with hype," says Arthur Caplan, a bioethicist at the University of Pennsylvania in Philadelphia. "If you read all the press releases, you'd presume that what you ought to do is prepare for immortality."

Yet it is clear that, despite a slow start to 2005, investors are backing the industry. According to an Ernst & Young report released this month, the revenues of US biotechnology companies grew by 17% from 2003 to 2004.

And back in Washington, what was arguably BIO's finest hour is feeding a sense of optimism. Last month, when the House of Representatives voted to loosen restraints on federal funding for human embryonic stem-cell research, several Republicans opposed to abortion voted for the bill.

"That's a very historically important moment," says Greenwood. "It means we can wrestle this issue away from the abortion issue and make it about hope and human health. It's the beginning of the beginning."

Coordinating vaccine use is best way to combat polio

SIR — In their Commentary article “A global call for new polio vaccines” (*Nature* **434**, 699; 2005), David L. Heymann, Roland W. Sutter and R. Bruce Aylward argued for vaccines to be developed and stockpiled. I propose, instead, that there is no need for new polio vaccines, but rather that more sensible and coordinated use is needed of the two major vaccines in existence.

One of these is the inactivated Salk vaccine and the other the live, but attenuated, Sabin vaccine (also known as oral polio vaccine). The Salk vaccine is safe, and was used to eradicate polio in Sweden. The Sabin vaccine is not so safe: for many years in the United States most of the polio cases that did occur were shown to have originated from the Sabin vaccine, owing to reversion of the attenuated viruses.

At the time both vaccines were introduced (a little over 50 years ago), I was an employee of the Lilly Research Laboratories of Eli Lilly and was intimately involved in production of the material for Salk's clinical trial.

Even in those days, I could not understand why a decision was never made, from a public health viewpoint, to insist that all populations slated for vaccination should receive one or possibly two shots of the inactivated Salk vaccine before receiving the live Sabin vaccine. The live vaccine has the advantage of multiplying in the gut, the normal site of polio multiplication, and thus triggering a greater reaction by the host's immune system. This normal intestinal route of infection was one reason swimming pools were considered to be such a hazard. However, if the host had first been protected by the Salk vaccine the risk posed by the Sabin dose would be reduced.

I still believe that this is more sensible from a public health viewpoint and, today, one no longer has to be concerned about the animosity between the vaccine developers or manufacturers.

Irving S. Johnson

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Don't drop current vaccine until we have new ones

SIR — David L. Heymann and colleagues, from the World Health Organization (WHO) issued a global call for new poliovirus vaccines in their recent Commentary article (*Nature* **434**, 699; 2005). This represents a welcome change in the WHO poliovirus eradication strategy; however, some important issues remain to be addressed.

The global struggle against poliomyelitis has been a huge success and, ultimately, is

expected to lead to eradication of the disease. However, several risk factors associated with the principal vaccine used today (oral polio vaccine, OPV), suggest that new vaccines will still be needed to accomplish eradication.

As Heymann and colleagues note, circulating vaccine-derived polioviruses have caused poliomyelitis outbreaks in five countries. Moreover, some people continue to excrete virulent poliovirus more than ten years after being vaccinated with OPV. Ending OPV immunization, therefore, can lead to increased risks from OPV derivatives, and Heymann and his colleagues are justified in calling for the development of new vaccines.

At present there are no adequate alternatives to trivalent OPV, but the WHO proposes to replace it with monovalent OPV (mOPV). Although mOPV vaccination may be useful under some circumstances, it poses the same risk factors as OPV. These risks vastly increase if mOPV is used in the post-vaccination era, because of diminishing population immunity.

“One no longer has to be concerned about animosity between vaccine manufacturers.” — Irving S. Johnson

Switching from OPV to the more costly Salk inactivated vaccine (IPV) eliminates these risks, and this has occurred in most industrialized countries. However, accidental release of the wild-type virus during IPV manufacture threatens to restart epidemics. Therefore, the WHO suggests using ‘Sabin IPV’ (sIPV) produced by inactivation of OPV. However, questions about the effectiveness of sIPV require further research and extensive clinical trials.

We propose instead that time should be given to conducting research for truly new poliovirus vaccines, and for the development of anti-polio drugs.

We also urge the WHO and public-health authorities around the world not to stop OPV vaccination until an efficacious and low-cost IPV has been developed.

We must also consider whether we should ever terminate poliovirus vaccination. In a world threatened with terrorism, we should remember that polioviruses could be synthesized rapidly at very low cost. The polio outbreak that happened in the 1940s in an Eskimo village in arctic Canada, with 25% poliomyelitis and high mortality, provides a sobering example of the devastation that can occur in unvaccinated communities.

Vadim I. Agol*, Konstantin Chumakov†, Ellie Ehrenfeld‡, Eckard Wimmer§

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Images: keep a distinction between beauty and truth

SIR — Your News Feature “CSI: cell biology” (*Nature* **434**, 952–953; 2005) addressed an insidious, but largely ignored, problem with undocumented image enhancement in scientific papers.

The enthusiasm for presenting the ‘best’ scientific image possible seems to be driven by a desire both to tell a clear story and to compose an aesthetically pleasing image. It would be helpful if all journals adopted a code of image-manipulation ethics, such as those described in the News Feature for *The Journal of Cell Biology*, to guide authors and reviewers alike.

After teaching extramural microscopy and imaging courses for a number of years, I have observed two additional factors that contribute to the widespread manipulation of scientific images.

First, graduate school curricula typically do not offer systematic instruction in microscopy or image formation, with the result that most biology graduate students rely on ad-hoc training by more senior students or postdocs. Without comprehensive training, many junior scientists are unable to produce the quality of image desired and resort to image software manipulation to ‘fix’ the image. Developing expertise in image acquisition would be preferable to resorting to post-acquisition manipulation.

Second, your News Feature attributed the increase in questionable image manipulation practices to the eagerness of students and postdocs to improve their data.

However, in my courses, many trainees report that they are instructed — often pressured — by the principal investigator to produce images consistent with expectations. This often means losing the dynamic signal range inherent in biological material to create a high-contrast ‘unambiguous’ image. Such instructed manipulation, either in image acquisition or post-processing, essentially discards data at best and may be misleading at worst. Thus it is incumbent, not only on the scientist in training, but also on the scientist performing the training, to maintain high ethical standards while pursuing both beauty and truth.

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COMMENTARY

Japan's whaling plan under scrutiny

Useful science or unregulated commercial whaling? **Nicholas J. Gales, Toshio Kasuya, Phillip J. Clapham and Robert L. Brownell Jr** consider the scientific merits of Japan's whaling activities.

Eighteen years after initiating scientific whaling in Antarctic waters, Japan presented a new and more ambitious programme to the International Whaling Commission (IWC); the proposal was made in early June during the IWC's annual meeting in Ulsan, Korea. Japan now wishes to more than double its annual catch of Antarctic minke whales (from about 440 to 935), and to expand lethal sampling to include an additional yearly take of 50 humpback and 50 fin whales. Unlike catches for commercial whaling, scientific catches are unregulated. Since 1987, Japan has taken some 6,800 minke whales from Antarctic waters, despite ongoing criticism of the relevance and direction of Japan's research.

The IWC was set up to regulate commercial whaling and to conserve whale populations, under the authority of the 1946 International Convention for the Regulation of Whaling. Following a well-documented failure of management that led to the collapse of most global whale populations, the IWC set a zero quota for commercial whaling (the moratorium). This was made effective from 1986. Norway, the former Soviet Union and Japan initially objected to the moratorium, but Japan withdrew its objection and ceased commercial whaling in 1988.

Scientific whaling occurs under Article VIII of the convention, whereby each member nation can grant its nationals a permit to take whales for scientific purposes. Unlike the international regulations on commercial and aboriginal/subsistence whaling, the objectives of the research and the number of whales to be killed for scientific purposes are set unilaterally by the member nation. Although the Scientific Committee (SC) of the IWC provides expert assessment of national research plans, the nations carrying out scientific whaling are not obliged to modify their research.

The first phase of Japan's scientific whaling commenced in the 1987–88 Antarctic season. In 1994, Japan also began scientific whaling operations in the western North Pacific, originally targeting minke whales, but subsequently expanding its catches to include Bryde's whales, sei whales and sperm whales. Since 1987, Japan has taken approximately 7,900 minke whales, 243 Bryde's whales, 140 sei whales and 38 sperm whales for scientific purposes. By contrast, 840 whales were killed globally by Japan

Since 1987, Japan has taken around 8,300 whales, including 38 sperm whales, for scientific purposes.

for scientific research between 1954 and the moratorium. Together, all other nations have killed about 2,100 whales for scientific research since 1952. Japan's expanded programme will result in annual catches that are more than half the total cumulative catches for scientific research by all nations in the past half-century. Such takes differ little in scale from commercial whaling, and must be justified by an adequate scientific rationale.

Conflicting opinion

The lethal sampling of whales for scientific research is extremely controversial^{1–4}. Many SC members (ourselves included) have consistently complained that such catches do not have sufficient scientific basis. The strongest scientific argument in favour of lethal sampling — the collection of genetic samples for determining population structure — could be conducted far more efficiently using non-lethal biopsy techniques. At the IWC meeting this month, a paper signed by 63 scientists representing 16 out of 30 national delegations contested the scientific claims of the Japanese proposal.

The tragedy for the scientists involved in the debate on scientific whaling is that they are labelled as either pro- or anti-whalers. This

impugns objectivity and relegates any discussion to polarized politics. As long as the whale catches remained small, the consequences of this gridlock were limited to political frustration. But with Japan's proposed escalation in the number of species and individual whales to be sampled, and without any regulatory process to manage these catches, the consequences for whale populations may well be more serious.

A 1997 IWC review of Japan's scientific whaling reported that the research conducted failed to meet its stated objectives and that the data derived were “not required for management”. Even today, the programme's publication record is very poor for an 18-year research endeavour of this size. Very few peer-reviewed papers have come from the Japanese programme, none has been published in the IWC's management-focused *Journal of Cetacean Research and Management*, and only one (on stock structure) is relevant to the scientific parameters used for species management.

A further criticism by SC members is that Japan's scientific whaling occurs within the IWC's Southern Ocean Whale Sanctuary where commercial whaling was specifically prohibited (so that scientists could study

IMAGE
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populations not subject to whaling). However, repeated calls by the IWC to Japan to halt its scientific whaling activities have had no effect.

At this year's IWC meeting, SC members were asked to provide an objective scientific assessment of Japan's new whaling proposal. This was to be done without the benefit of an independent review of Japan's previous 18 years of Antarctic scientific whaling, and with the knowledge that there is no mechanism in the convention to ensure that Japan responds to any review process.

Looking back

Japan intends to proceed with its expanded whaling programme before an independent review is completed (scheduled for late 2006). So it is important to examine how this second phase of whaling differs from the first 18-year phase, and how realistic or relevant Japan's new objectives are. Although there is no time limit to this second phase, in an equivalent 18-year period Japan can be expected to take about 17,000 minke whales, 820 fin whales and 800 humpback whales from the Southern Ocean. This will be in addition to increased catches in the western North Pacific.

During its first 18-year phase of whaling, Japan's main scientific objective in the Antarctic was to improve estimates of minke whale population parameters (such as age-specific mortality rates), which, Japanese scientists argued, were needed for effective management of the whales. Yet previous IWC management failures have been attributed to problems with such data³, and procedures no longer require it. Despite this, Japan continues to include this objective in its plans. A notable addition to Japan's aims is to manipulate the ecosystem through selective culling of certain species, with the explicit intention of reducing interspecific competition and thus promoting population growth in the most economically valuable species (such as blue whales).

At the heart of Japan's new proposal is their hypothesis that whales are competing directly for a limited resource (krill). Ignoring the fact that current whale populations, and thus their collective consumption of prey, remain at fractions of pre-whaling levels, Japan postulates that the recovery of depleted blue whales will be negatively affected by population increases of humpback, fin and minke whales (although data on abundance and population trends for all species are highly uncertain or non-existent). This hypothesis has been proposed using primarily unreviewed and unpublished data collected during the first 18-year phase of scientific whaling. Moreover, Japan proposes using a crude ecosystem approach to examine this hypothesis. This includes constructing simplistic models of competition among whale species, and inadequately measuring

IMAGE UNAVAILABLE FOR COPYRIGHT REASONS

Strong opposition: demonstrators in South Korea object to Japan's new whaling proposal.

other components of the Southern Ocean ecosystem including krill abundance and habitat features.

A better understanding of the Southern Ocean ecosystem is critical to considerations far beyond the management of whales. Oceanography, and studies addressing climate change and fishery management have led to a series of successful multi-disciplinary, multinational collaborations. The Convention on the Conservation of Antarctic Marine Living Resources (CCAMLR), to which Japan is a signatory, applies an ecosystem approach to the conservation and rational use of the Southern Ocean's living resources (primarily krill and fish). To this end, CCAMLR's members have a strong history of ecosystem research, and of developing ecosystem models. Studying the biomass and dynamics of krill and krill predator populations (including whales, the data on which come from the IWC) are within the mandate of CCAMLR. In contrast, Japan's proposal to unilaterally conduct its whale-focused ecosystem-scale research, isolate it from the benefits of multi-disciplinary scientific input and collaboration.

From a conservation perspective, Japan's planned catches of humpback and fin whales in the Southern Ocean are particularly worrying. Humpback whales are listed internationally as vulnerable and fin whales as endangered — heavy exploitation in the twentieth century saw total Southern Hemisphere catches of 723,000 fin whales and 197,000 humpbacks. The species have been protected from any form of legal whaling in this hemisphere since 1966 (humpbacks) and 1985 (fin whales).

Very little is known about the status of fin whales in the Southern Ocean. But some of the humpback whales feeding where Japan

intends to conduct whaling come from small, highly depleted populations that breed in the tropical South Pacific. Because gunners on catcher boats cannot know the population from which a particular whale is taken, catches in these regions could have disastrous effects in terms of stock recovery for these populations.

Up for review

It is time for the IWC to review the provisions of the International Convention under which scientific whaling permits are issued. Science is stipulated as the basis of management procedures within the IWC. But the lack of a science-based regulatory process to manage scientific whaling, and the escalation of this whaling to commercial scales on the basis of poorly established and controversial scientific claims, challenge the idea that the IWC can deliver a robust framework for whale conservation or a sustainable whaling industry.

The SC must be given a real role in determining the IWC's scientific needs, the best methods to achieve these needs, and what risks such research might pose to the conservation of whale populations. The minimum regulations applied to any proposed lethal catches made for scientific purposes, should they be accepted by the SC, must equal those applied to commercial whaling. Furthermore, if commercial whaling resumes, any lethal catches must be part of future national quotas.

Japan's scientific whaling programme yields considerable annual revenue from the commercial sale of whale meat, estimated at US\$50 million earlier this decade; this will rise considerably as catches increase. The Japanese government provides annual subsidies of some further US\$10 million for cetacean research. These revenues are invested in the maintenance and operation of the catcher/processor vessels in addition to the Japanese Institute of Cetacean Research that conducts the science associated with scientific whaling. The risk for Japan is that dependence upon these revenues could drive its quotas for scientific whaling, yet leave the real scientific questions unaddressed. ■

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BOOKS & ARTS

Crash and burn

Even 'artificial organisms' such as commercial companies find immortality out of reach.

Why Most Things Fail: Evolution, Extinction and Economics

by Paul Ormerod

Faber & Faber: 2005. 272 pp. £12.99

Adrian Woolfson

In *An Enquiry Concerning Political Justice* (1793), social philosopher William Godwin argued that it should be possible to extend human life indefinitely through "the sway of mind over matter". His recipe for immortality included the cultivation of benevolent and optimistic attitudes. Alchemists also sought to deliver the secrets of longevity but, frustrated by their failure, and refusing to be thwarted by their own finitude, humans sought other ways of imprinting themselves on the future. The pharaohs built pyramids, and the Mongolian tyrant Genghis Khan conquered vast tracts of Asia and Europe, reputedly siring so many offspring in the process that as many as 1 in 200 men may be descended from him.

But if flesh could not be immortalized, why not imitate it, creating artificial beings capable of indefinite existence? In *Leviathan* (1651), the philosopher Thomas Hobbes suggested that the nation state is a type of 'artificial man'. Ancient Greece and Rome had already spawned their own type of artificial being: the corporate entity. Greek *etairia* corresponded closely to modern corporations, and Roman *collegia* enabled property to be held in common. Medieval European business enterprises, such as the early Italian banking firms, were the forerunners of modern multinational companies.

The pivotal event in the evolution of modern corporations came in 1811, when New York state filed legislation enshrining the principle of limited shareholder liability; until this point, investors holding even a single share in a company were liable for unlimited losses. But with the adoption of this legislation and the resulting injection of low-risk capital, new companies flourished and New York City became the world's premier financial centre. Other nations followed New York's lead, giving rise to modern stock markets and the global economy.

In his interesting and entertaining book *Why Most Things Fail*, Paul Ormerod explains why this experiment with artificial immortality was fatally flawed. Like natural species, companies walk a fine line between existence

Boom and bust: the New York Stock Exchange has witnessed the demise of countless companies.

and extinction. The first biological species on Earth emerged some 3.45 billion years ago. Organisms remained pretty simple until complex multicellular life erupted 550 million years ago during the Cambrian explosion. This was when all modern phyla were formed, as well as alternative animal designs that left no descendants. Ormerod suggests that this burst of biological creativity was mirrored in the 'Edwardian explosion' of 1880–1910, which witnessed the emergence of the first truly multinational corporate entities. By the start of the twentieth century, for example, US Steel employed more than 20,000 people and in 1917 had assets in excess of \$2.4 million (\$400 billion in today's terms). But like most of the Cambrian phyla, many of the new corporations became extinct. Neil Fligstein noted in *The Transformation of Corporate Control* (Harvard University Press, 1990) that only 33 of the top 100 US companies of 1912 were still in the list in 1979. Artificial corporate organisms are like their flesh-endowed counterparts, both fallible and mortal. Indeed, each year more than 10% of all US companies disappear.

So why do most companies fail — and can management consultants, economists and business gurus do anything to reverse this

apparently inexorable trend? Ormerod examines a host of complex systems, including societies, corporations, species, ecologies and government social policies. Can failure in such diverse systems be explained by a general theory? This is an unnerving suggestion, as the prediction of uncertain futures is far from easy. Consider, for example, the fundamental indeterminacy at the subatomic level described by quantum mechanics. Chaos theory tells us that small changes to the starting conditions can have immense consequences, and the theory of computation suggests that there may be no faster way of determining the behaviour of a non-equilibrium system than watching it unfold.

Ormerod attributes the failure to predict phenomena as diverse as the demise of the blue-chip companies Enron and WorldCom, the failure of Coca-Cola's 'New Coke' in the 1980s, and the collapse of the Soviet Union to an outdated analytical methodology. Traditional 'general equilibrium theory' economics envisages a platonic ideal in which companies are systems in equilibrium with perfect access to information and an unlimited ability to analyse it. Agents act rationally, landscapes are static, and the state of the system can be computed using differential calculus. Recent

IMAGE
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modifications incorporating game theory or bounded rationality are no different.

These methods have largely failed, and it was discovered that the five great periods of biological extinction, the inventory of failed multinational companies, and phenomena as diverse as stock-market crashes, biological phenomena and the structure of contacts on the World Wide Web are described by a 'power law'. Clearly, deeper forces are at work. In a power law, the frequency of an event falls away with the square of its size. These causal factors emerge not from outside perturbations, but from the intrinsic dynamics of highly interconnected networks that are far from equilibrium. The fascinating generic behaviour of these networks and the generation of complex behaviour from the iteration of simple rules has been beautifully described by Stuart Kauffman and Stephen Wolfram. So it is a shame that Ormerod does not discuss how the

invisible hand of emergent network behaviour makes its presence felt.

There are some gems nevertheless, and the scale and breadth of Ormerod's analysis deserves commendation. Most interesting is the way in which power laws challenge conventional notions of causality. The stock-market crash of September 1987, for example, in which the Dow Jones index collapsed by 20% in a single day, may not ultimately have had a distinct cause, as catastrophic events may occasionally have insignificant causes. More important, within Ormerod's framework, successful institutions evolve organically, indicating that excessive government intervention may be both unnecessary and counterproductive. ■

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when the infection moved slowly from rural towns to the provincial capital before quickly spreading out from the province to the rest of China, Hong Kong and much of the world.

This is what we could not know when Chinese officials hid the facts despite incessant internal and external enquiries from the public, media, health agencies, governments and the WHO. At last, we learn how SARS first appeared and spread inside Guangdong, and how the local health system responded, with many examples of professional dedication, clinical excellence, intelligent investigation and selfless behaviour. Guangdong doctors produced excellent guidelines on diagnosis, treatment, hospital infection control and quarantine as early as 23 January 2003 — six weeks before the global emergency erupted. This information could have prevented thousands of infections but was not shared, even within China. Abraham gives us an excellent and dispassionate account of the cultural and political background to the cover-up, and the unfortunate consequences, both epidemiologically and politically, for China. There are lessons for everyone, as all governments could be tempted to cover up infection outbreaks, especially if the rest of the world cuts off social and economic contact. Transparency was the only solution for SARS, and eventually China paid a high price for its initial secrecy.

Subsequent chapters cover more familiar ground: the introduction of the disease to Hong Kong through an infected doctor visiting from Guangdong; explosive local transmission in several hospitals and a high-rise housing complex; and the spread to Singapore, Vietnam, Canada, Beijing and Taiwan. We also learn about the hunt for the virus and the crucial role of the WHO in inducing international collaboration and steering the global control efforts for this terrifying epidemic.

Politics and disease

Twenty-First Century Plague: The Story of SARS

by Thomas Abraham

Johns Hopkins University Press: 2005.
176 pp. \$24.95

Adrian Sleight

As a new and lethal disease caused by a previously unknown virus, severe acute respiratory syndrome (SARS) was the first species-jumping global epidemic this century, and will not be the last. This slim book puts SARS in context with an opening chapter on emerging infections, and ends with one that considers the looming threat of pandemic influenza.

Twenty-First Century Plague is an accurate and intriguing account of the complexities of the SARS story, the interacting political responses and the underlying bioepidemiology, involving primarily China, Hong Kong and the World Health Organization (WHO). Written by Thomas Abraham, a journalist based in Hong Kong, the book focuses on the main players. We get a ringside seat to an accessible, well-referenced account of the science, the politics and the people involved.

The SARS story begins in China, and Abraham starts by illuminating the least-known aspect — what happened in Guangdong from November 2002 to March 2003. That was

IMAGE
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We see the costs and benefits of our globalized world — at higher risk, but mobilizing faster and more smartly than ever before. This was the first 'digital' response to a lethal global epidemic: there were many examples of new digital tools, such as some excellent case-information and geographical contact-tracing systems devised in Hong Kong and a secure Internet-linked laboratory network mounted by the WHO.

Discovering and characterizing the causal virus and developing diagnostic tools transformed the global battle against SARS, and was done faster than for any previous infection. The tension created by simultaneous competition and collaboration among scientists chasing the virus, and the balance needed between excessive caution and reckless haste, are well described. This makes the virus hunt one of the most fascinating aspects of the SARS story. There were false leads and bad luck with initial culture attempts, but eventual success for several groups. Hong Kong University came out just ahead in the international race to identify the causative agent, and its findings were validated by others within a few hours. This satisfactory scientific outcome contrasts with that experienced by the hapless

young Chinese scientists who seem to have discovered the virus several weeks earlier but could not reveal their findings because they would have contradicted incorrect statements made by their superiors — yet another price paid by China for its initial management of SARS.

The role played by the WHO is also an excellent story by itself. A small team based in Geneva, Manila, China and Vietnam collaborated to provide the global and regional leadership needed, and the results were excellent. We find out how difficult the task was — involving political skill, judgement and courage. It is clear that the WHO in 2003 had just the right people to meet the SARS challenge. Unfortunately, we cannot be assured that such resolve or skill will appear next time, and this is one of the more sobering lessons from SARS. If left chronically underfunded, as in the past, the WHO may not have the staff and vigilance needed next time a comparable challenge arises.

I commend this book to those with an interest in emerging infections, SARS and China. ■ Adrian Sleigh is at the National Centre for Epidemiology and Population Health, Australian National University, Canberra 0200, Australia.

collections, will remove the need for much *in situ* bioprospecting. And combinatorial chemistry will replace the need to find resources in the wild.

Refreshingly, Parry contests the frequently posited position that combinatorial chemistry is free of all benefit-sharing obligations, and notes the need to close the legal loophole regarding pre-CBD collections. One key conclusion is the need to develop effective means of tracing the many and extensive uses of genetic resources and derived informational products. Unfortunately, her study does not refer to the work of the CBD in this area, in particular to the ongoing study of the practicalities, feasibility and cost of certificates of origin as a means of tracing the flow of genetic resources. I would also have liked to see more in-depth analysis of potential technological means of responding to the challenges of resource transformation and the monitoring of direct and indirect uses of genetic information.

Parry comes to the conclusion that further efforts to secure regulatory control are misplaced, suggesting instead that countries should seek alternative benefit-sharing mechanisms. She also suggests the establishment of an international trust for benefit sharing, an idea that is to some extent akin to the model included in the recently adopted International Treaty on Plant Genetic Resources for Food and Agriculture of the United Nations Food and Agriculture Organization. However, her failure to expand on this area of research and place it within the context of the ongoing ABS negotiation process weakens the value of what could have been a more substantial and reasoned proposal.

Overall, however, the book is a welcome addition to the literature and will be a valuable resource for researchers, decision-makers and members of the public who are interested in understanding what happens to genetic resources after they have been collected. It will provide little in the way of policy insight for veterans of the bioprospecting debate, but its organized presentation of the transformation of the bioprospecting trade and of the development of bioinformatics is well worth the read. However, its conclusions need to be considered in the light of recent developments. Among these are efforts by developed countries to adopt so-called 'user measures' to regulate the use of imported genes; debates in the CBD, the World Intellectual Property Organization and the World Trade Organization on proposals to include obligations to disclose the origin of genetic resources in patent applications; and work examining the feasibility, practicality and cost of mechanisms, such as certificates of origin to track flows of genetic resources, as described in a United Nations University report (www.ias.unu.edu/binaries/UNUIAS_UserMeasures_2ndEd.pdf). ■ Brendan Tobin is at the Institute of Advanced Studies, United Nations University, Nishi-ku, Yokohama 220-0012, Japan.

The power of collecting

Trading the Genome

by Bronwyn Parry

Columbia University Press: 2004. 352 pp.
\$39.50, £25.50

Brendan Tobin

The collection, control and commodification of biological resources, historically seen as the common heritage of humanity, have long had a role in the efforts of nations to build and maintain their economic and political power. One of the earliest and most ambitious projects of this sort was the proposal made to Britain's King George III to transplant breadfruit from Tahiti to serve as a source of food for slaves in the West Indies. A mutiny on the ship chosen to transport the plants — the *Bounty* — quickly brought to an end the first major state-supported attempt by the scientific community to relocate economic plants.

More recently, advances in biotechnology and information technology have transformed the processes involved in the collection, transport and storage of biological resources and the information they contain. A new surge in collection in the 1980s was fuelled by improvements in the ability to isolate compounds from plants and animals, coupled with regulatory mechanisms enabling patents to be obtained over them. This time, however, developing countries, the primary source of biological resources, strongly resisted what some saw as biocolonialism. The United

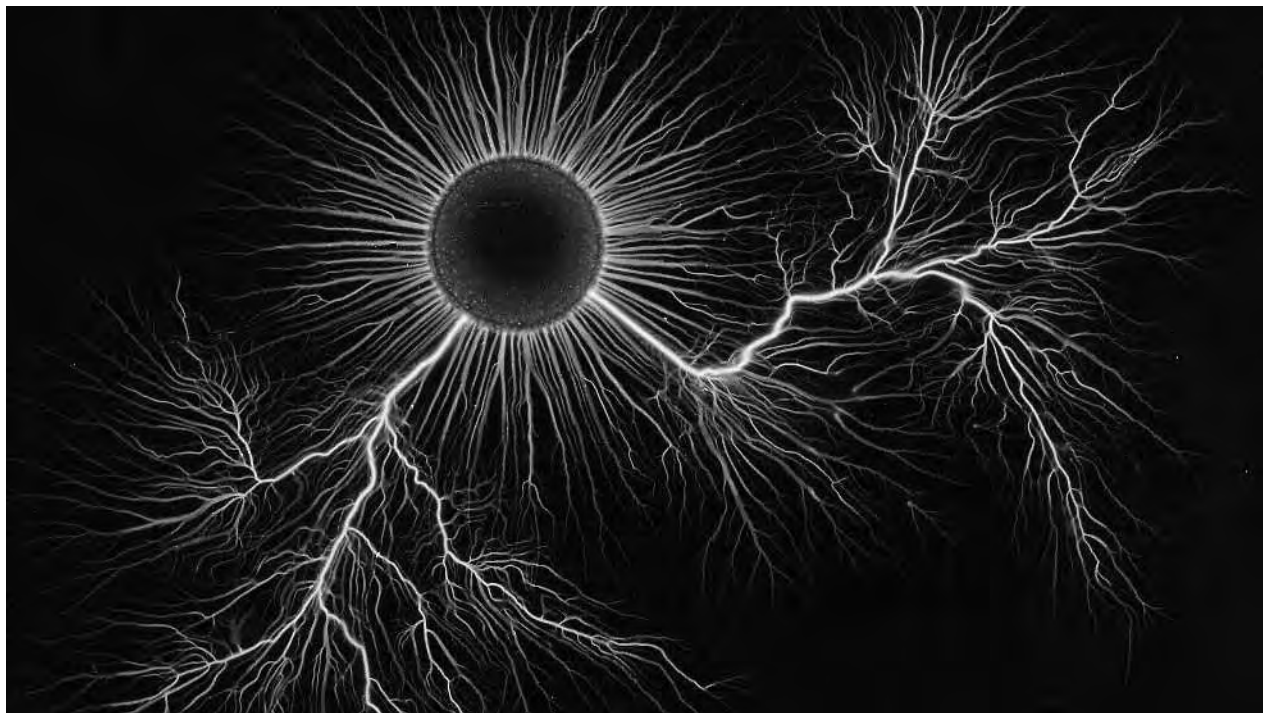
Nations Convention on Biological Diversity (CBD) of 1992 was intended to respond to this conflict and ensure the equitable sharing of benefits derived from the access to, and use of, genetic resources. But the convention has so far had only limited success, and at the World Summit for Sustainable Development in Johannesburg in 2002, calls were made for the negotiation of an international regime on access and benefit sharing (ABS).

The history of biodiversity collection, and the transformation and commodification of genetic material, is the subject of *Trading the Genome* by Bronwyn Parry. Reviewing both the technological and the regulatory history of the gene trade, Parry's book provides insight into the complex problems facing the global community in regulating the access to, and the use of, genetic resources. With negotiations for the development of an international ABS regime due to begin in earnest in January 2006 in Spain, this publication is particularly timely.

Focusing her research on a number of key US initiatives, including those involving the National Cancer Institute and the US pharmaceutical industry, Parry reviews their collection, research and product-development practices, and long-term objectives. She persuasively argues that the end of large-scale bioprospecting is drawing close. Ever more sophisticated means of synthesizing and replicating collected material, as well as of mining both living and dried specimens in *ex situ*

Trees of knowledge

Georg Lichtenberg visualized a new branch of science.



A. VON HIPPEL

Martin Kemp

There is something compelling about a phenomenon that can inscribe itself, drawing its own diagram through the direct visual recording of traces of its activity. We are familiar with these kinds of traces in the cloud and bubble chambers of atomic science, but the idea that the unseen forces of nature might reveal their actions through visible traces has a much longer history.

Acoustics was probably the first field to visualize such forces. As early as 1680, Robert Hooke studied wave formations in flour on glass plates agitated by a violin bow. This began a tradition of materializing sound that passed via Ernst Chladni in 1787 and Hermann von Helmholtz to modern oscilloscopes.

But nowhere were the results of direct transcription more spectacular than in the new science of electricity in the late eighteenth century. Working at the University of Göttingen, Germany, as professor of physics, Georg Christoph Lichtenberg stumbled across the explosively beautiful dendritic structures of electrical discharge that still bear his name. The activities of this humorist, polemicist and moralist ranged from the mathematical sciences and physiognomics to commentaries on William Hogarth's satirical engravings. It was while he was experimenting with static electricity on a huge cake of resin that he discovered Lichtenberg figures, partly by chance. He was fascinated to observe that dust

had accumulated into a radiating formation of startling refinement and complexity.

The direct inscription of Lichtenberg figures on to a photographic plate was accomplished by Arthur von Hippel and his student Fred Merrill in the late 1930s. They set up an impulse generator and pressure tank so that they could record photographically the effect of an electrical discharge in any gas over a wide range of pressures. The resulting images (see above), as von Hippel realized, have extraordinary visual properties. Neatly capturing the nature of lightning, the photographs "transfer terror into enchantment", as Lichtenberg put it, and manifest art in science.

Four of the Lichtenberg figures obtained by von Hippel and Merrill feature in a fascinating exhibition exploring the ramifications of dendritic structures, *Einfach Komplex (Simply Complex)*. The exhibition, curated by Barbara Bader, Andres Janser and Marius Kwint, can be seen at the Museum of Design in Zurich, Switzerland, until 4 September. Featuring images of trees in science, it not only presents us with a visual feast of naturally occurring structures that provide variations on fractal branching systems, and with works of artists and designers who are fascinated by dendritic formations, but also encourages us to journey into the conceptual branches of the organization of knowledge.

As the curators show, tree-shaped 'organograms' have provided schematic

forms for the organization of intellectual fields, encyclopedias, digitized data, languages, artistic styles, biological evolution, voter choice and much more. Few of us who work in large organizations can have escaped being located somewhere on a ramifying chart of line management and accountability.

What the physically generated forms (such as the Lichtenberg figures) and the conceptual models generally have in common is a basic rule of branching structures: thick branches tend to give rise to progressively thinner ones in a proportional manner. Lichtenberg himself sensed some kind of affinity between the 'folds' of his thought and the tendrils of his figures.

But Lichtenberg would probably have resisted the schematization of thinking that can too easily accompany the condensing of complex phenomena into diagrams of branching structures, just as he rejected the easy formulas of physiognomics for judging someone's character. He was well aware of the sheer complexity of nature, human character and society. As one of the greatest ever writers of aphorisms, it is fitting that he should have the last word: "The noble simplicity in the works of nature only too often originates in the noble short-sightedness of he who observes it." Martin Kemp is professor of the history of art at the University of Oxford, Oxford OX1 1PT, UK, and is the author of *Leonardo* (Oxford University Press, 2004).

LOW-TEMPERATURE PHYSICS

Tunnelling into the chill

Jukka Pekola

The trend towards ever smaller electronic instruments had left refrigerators out in the cold. Now a practical, compact device uses quantum mechanical tunnelling to cool close to absolute zero.

The French physicist Jean Peltier discovered in 1834 that when an electrical current is passed through a solid-state circuit, heat is in some cases removed. Yet one obvious application, a solid-state micro-refrigerator capable of cooling to cryogenic millikelvin temperatures, has remained science fiction. Writing in *Applied Physics Letters*, Clark *et al.*¹ report significant progress in constructing such a device.

Several sophisticated astronomical and analytical instruments rely on thin-film sensors that must be cooled to temperatures of 0.1 K or lower. One example is the satellite-based, ultrasensitive radiation detectors that are being used in the search for anisotropy — tiny temperature fluctuations — in the cosmic microwave background thought to be leftover radiation from the Big Bang. Advances in microfabrication mean that such sensors can be very small and light — an obvious advantage in space-borne astronomical instruments. So the required combination of small bulk and low-temperature operation highlights the need for miniaturized refrigerators. In 1994, Nahum *et al.*² demonstrated the so-called NIS refrigeration effect using a metallic thin-film device, and since then progress has been rapid. Clark *et al.*¹ now supply a first practical device based on this effect.

The principle of NIS refrigeration is simple (Fig. 1): a normal metal (N) is separated by an insulating (I) barrier from a superconductor (S). Electrons can pass across the insulating barrier only by quantum tunnelling — a consequence of the uncertainty in quantum mechanics that means there is a finite probability of finding a particle on the other side of a barrier, even if, in terms of classical physics, it does not possess enough energy to surmount that barrier. Cooling occurs as a consequence of the different electron configurations in the N and S regions: in the normal metal, electrons occupy states with an almost constant density over the whole range of relevant energies, whereas in the superconductor there is a gap in which no electron energy states exist. Electrons in the normal metal at energies corresponding to the gap in the superconductor are forbidden from tunnelling through the insulating barrier.

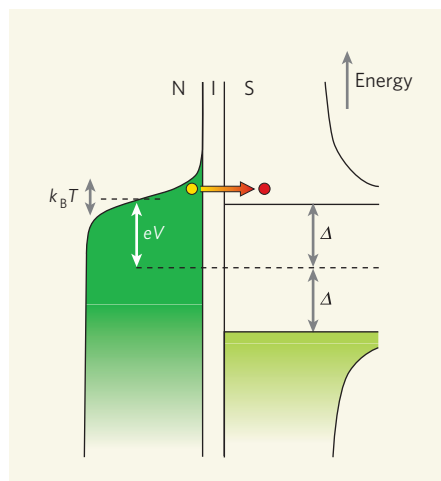


Figure 1 | The working principle of the NIS micro-refrigerator devised by Clark *et al.*¹. In a normal metal (N), energy states are filled with electrons at almost a constant density up to the chemical potential, which represents the top of the occupied energies 'smeared' by an amount $k_B T$, where k_B is the Boltzmann constant and T is the temperature in N. The electronic structure of a superconductor (S) differs fundamentally from that of a normal metal in that it possesses an energy gap of width 2Δ in which no electron energy states are found. At very low temperatures, states in S are perfectly filled below the gap and empty above it. Applying a voltage V to the NIS system causes the chemical potential (dotted line) of the metal to shift relative to that of the superconductor by an amount eV (e is the charge on an electron). If V is sufficiently high, the most energetic filled states in N correspond to empty, but allowed states in S, enabling quantum tunnelling of electrons from N to S through the insulating barrier (I) to take place. The energy of the electron system left behind in N is therefore reduced, resulting in cooling.

In the absence of an external voltage, the N and S regions are in thermodynamic equilibrium, with their zero energy points ('chemical potentials') aligned — in the middle of the energy gap in S (Fig. 1). Applying a voltage V across the barrier shifts the chemical potential of N relative to that of S. At low temperature and voltage, the relative shift of the zero levels is not sufficient to allow electric current to

flow between N and S, as the energy of the occupied states in N still corresponds either to forbidden states in the gap, or to occupied states below the energy gap in S — thus quantum tunnelling remains forbidden. But at higher voltages, as the energy shift in N approaches Δ , half of the energy gap in S, current suddenly starts to flow owing to the vertical matching of the most energetic occupied states in N and the empty, but allowed, states above the energy gap in S. As only the most energetic electrons are free to tunnel, the electron gas left behind has a lower average energy than existed before tunnelling — thus, the electron system in N cools down.

Cooling of a micrometre-scale, thin-film copper bar from 300 millikelvin down to 100 mK using a double-junction NIS device was demonstrated in 1996, and temperatures below 50 mK were reached last year^{3,4}. However, the cooling power in these experiments was low — typically of the order of one picowatt (10^{-12} W) — insufficient to cool astronomical detectors, where the background radiation typically exceeds this level. The cooling power has since been increased by almost two orders of magnitude by scaling up the physical dimensions of the refrigerator^{5,6}.

In most applications, however, chilling the electrons alone is not enough: heat must also be removed from the platform that houses the detector (or sample) to be cooled. One way to do this is to thermally isolate a dielectric platform (typically, a thin membrane of silicon nitride) by micromachining techniques, and to suck the heat from it to the cooled electrons in the NIS refrigerator. This approach has previously been used⁷ to reduce the temperature of such an insulating apparatus by a factor of two, from 200 to 100 mK.

New processes and different combinations of materials have since enhanced the cooling power of NIS refrigerators still further⁶. Clark *et al.*¹ incorporate these techniques into a full refrigerator, and test its cooling power on a 'macroscopic' germanium resistance thermometer in the form of a 250- μm -sided cube glued onto a silicon nitride membrane. Their device can reduce the temperature of this system significantly below that of its

surroundings — from 320 to 220 mK. The authors are thus the first to refrigerate a separate 'bulky', three-dimensional object, using an electronic method, down close to absolute zero. And they believe that more effective heat removal from the hot side of the refrigerator and the use of numerous cooler elements in parallel will allow them to improve both the attainable temperature reduction and the surplus cooling power.

The work of Clark *et al.* is an exciting development towards a fully solid-state, cryogen-free micro-refrigerator, which could eventually cover temperatures from the ambient down to the millikelvin range. Such an achievement would have an enormous impact in overcoming the 'cryophobia' that at present prevents the large-scale use of many devices

and sensors that can operate only at very low temperatures.

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GENETICS

LINEs in mind

Eric M. Ostertag and Haig H. Kazazian Jr

At least half the human genome consists of mobile elements, such as LINEs, some of which can jump around the genome. These elements have been crucial in genome evolution, but they may also contribute to human diversity.

Barbara McClintock won the Nobel Prize in Physiology or Medicine in 1983 for predicting the existence of mobile elements, pieces of DNA that move from one place in the genome to another. McClintock called them 'controlling elements' and proposed that they could account for developmental differences among individuals of a species — explaining, for example, the differences in maize-kernel colour that she observed¹. Although her ideas were not well received at the time, they have proven to be remarkably prescient. On page 903 of this issue, Muotri *et al.*² provide evidence that mammalian mobile elements may have a role in creating "the uniqueness of individuals within a population".

Mobile elements, also called jumping genes, exist in all living things, but the 'long interspersed nucleotide element-1' (LINE-1, or L1 for short) is the only active human mobile element. This element encodes the machinery to move itself and to mobilize other elements³. Muotri *et al.* demonstrate that a human L1 is active in both cultured rat neural progenitor cells (NPCs) and the NPCs of a transgenic mouse. Remarkably, *de novo* L1 insertions in cultured NPCs sometimes alter the expression of neuronal genes, thereby affecting NPC differentiation (the process by which the cells mature into specialized cells).

If L1 insertion occurs in the NPCs of developing human neuronal cells, then some cells will contain *de novo* insertions. The authors cautiously speculate that activity of mobile elements might be responsible for creating some

of the diversity among people. For example, if enough mobile DNA insertions occur in the brains of developing humans, then the outcome might be a change in their neuronal circuitry, for better or for worse.

One surprising find from this work is that L1 elements are actually active in NPCs. These elements are considered by many researchers to be no more than genetic parasites. From an evolutionary standpoint, L1 elements should be most successful if their expression is restricted to germ cells (sperm and oocytes), or to stem cells early in development, because mobility in these cell types will lead to an expansion in the number of L1 elements. Conversely, *de novo* L1 insertions in other cell types (somatic cells) cannot be passed on to future generations, and, if detrimental to host reproduction, would harm the chances of L1 propagation.

In fact, previous studies of L1 expression and mobility demonstrate L1 activity in germ cells and in early developmental cells^{4–6}, but not in other cell types. There has been only one previous example of L1 mobility in a human somatic cell: this insertion disrupts a gene that contributes to colon cancer⁷. Muotri *et al.* provide the first evidence of L1 activity in normal cells cultured directly from an animal sample, and the first evidence of somatic L1 activity late in development of a transgenic mouse.

The expression of L1s in NPCs appears to be inversely correlated with the expression of SOX2, a gene that is poorly expressed in developing NPCs but that has several vital functions

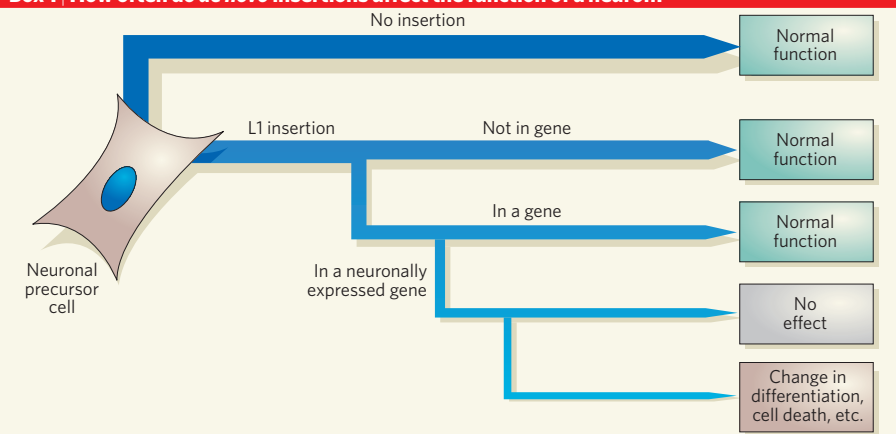
in adult neural cells. The authors further demonstrate that L1 activation is related to changes in histone proteins that are associated with gene expression in general. Histones interact directly with DNA, and their acetylation and methylation pattern can determine whether a region of DNA is 'open' and transcribed⁸. That histone modification might be a host mechanism to control L1 activity is an intriguing possibility.

Also a surprise is the extent to which *de novo* L1 insertions affect NPC development. An analysis of mammalian genomes demonstrates that L1 elements insert more or less randomly, landing both within and outside genes^{9,10}. The authors have not analysed enough *de novo* insertions from cultured NPCs to achieve statistical significance, but there seems to be a striking preference for insertions into or near genes. This particularly applies to genes that are neuronally expressed — in two cases (out of 17) insertions occur in the same neuronally expressed gene. Even more interesting is the finding that NPC differentiation is affected by L1 insertions. The researchers convincingly demonstrate that one such insertion into the *Psd-93* gene increases the expression of that gene, which in turn induces neural differentiation.

Why is there a discrepancy between the random insertion pattern of L1 elements previously found in the genome, and the apparent non-random insertion pattern observed in cultured NPCs? One possibility is that 'open' areas of DNA are more accessible for L1 insertion, and perhaps there are fewer 'open' areas in NPCs than in germ cells. Neuronally expressed genes are by definition 'open' in neuronal cells, and may be preferred sites for new insertions. Future experiments should determine whether the non-random insertion pattern also occurs *in vivo* or is peculiar to cultured cells.

Muotri *et al.*² hypothesize that mobile elements could affect neuronal development and diversity of brain function in humans, but there are several questions to be answered before this can be accepted. First, is the frequency of *de novo* insertions high enough to affect neuronal function? Most NPCs are not expected to contain *de novo* insertions, and many NPC insertions will have no effect on neuronal function (Box 1). Considering the predicted rarity of these events *in vivo*, the effects of mobile elements on neurons may be limited. That said, the authors did find several examples of these events in cultured cells.

Second, is the ability of L1 to move in NPCs a random quirk of nature? Or is this an evolutionarily maintained mechanism that creates individual variation? At first glance, it would seem that this process cannot be evolutionarily maintained. Any insertions that occur in NPCs are not passed on in the germ line, so any changes that insertions make to an individual are eliminated with each generation.

Box 1 | How often do *de novo* insertions affect the function of a neuron?

It is impossible to determine directly the frequency of insertions that occur *in vivo* in human neuronal precursor cells (NPCs), and Muotri *et al.*² were not able to quantify this number in transgenic mice. It seems, from stained brain sections, that insertions do not occur more often than once in 10 cells, perhaps only occurring as rarely as once in 1,000 cells. Although a small fraction of cells may appear to have new insertions, it is difficult to extrapolate the activity of an unknown number of highly active L1 elements in a transgenic mouse to the activity of a number of endogenous L1 elements. Most insertions will not occur in genes. If L1

insertion is entirely random, the percentage of insertions into genes would be about 30% — the percentage of the genome that is made up of genes (introns plus exons). Muotri *et al.*² have reason to believe that the percentage of gene insertions may be higher than 30% because of non-random insertion. However, to affect neural function, an insertion must occur in a neuronally expressed gene, and the insertion must have an effect on cell fate even when only one of the two copies of a gene is disrupted. Insertions into a single copy of some neuronally expressed genes may have no effect. **E.M.O. & H.H.K.**

However, if the mechanism to create diversity is encoded in the germ line (for example, by the number and activity level of mobile elements), and if diversity is favoured by natural selection, then this mechanism can be maintained through evolution.

Time and further research will determine whether McClintock's hypothesis that mobile elements have a significant role in an organism's development can be extended from maize to humans, and specifically to the function of human neurons.

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MALARIA

Fungal allies enlisted

Yannis Michalakis and François Renaud

The mosquito-killing capabilities of fungi can in principle be deployed in the fight against malaria. But long experience of unfulfilled hopes in this complex arena shows the need to proceed cautiously.

Many malaria control measures have centred on the mosquito vector of the *Plasmodium* parasite that causes the disease. Female mosquitoes transmit *Plasmodium* from human to human after feeding on the blood of an infected person — hence the age-old use of bednets, and the more recent attempts to genetically manipulate mosquitoes to make them resistant to parasite infection, or to reduce mosquito populations with insecticides. Two papers in *Science*, by Blanford *et al.*¹ and Scholte *et al.*², describe another approach — the deployment of mosquito-killing fungi.

Using mouse malaria as a model system, Blanford and colleagues¹ studied the effects of various isolates of these fungi on mosquitoes. Many isolates induced mosquito mortality of more than 80% within 14 days of infection, a period that corresponds to that in which *Plasmodium* produce offspring in the mosquito that are transmissible to humans. Further experiments with one of the fungal isolates, chosen because it is part of an existing

agricultural pesticide, showed that the fungi also have a direct effect on the development of *Plasmodium* in mosquitoes: only 8% of mosquitoes infected with both the parasite and fungi contained transmissible parasite offspring 14 days after exposure to the fungi, compared with 35% infected with *Plasmodium* alone. Putting the effects on mosquitoes and *Plasmodium* development together, fungi could reduce malaria transmission by approximately 80-fold. The effect might be even greater, given that fungal infection also decreases the propensity of infected females to feed on blood.

Blanford *et al.* carried out their experiments with several isolates of two different species of fungi and several malaria clones. They also tested various fungus-containing formulations, applied on nets or solid surfaces for different exposure times, and showed that their conclusions still held. Nonetheless, mouse malaria may have different characteristics from human malaria, and many different

factors can come into play when applying research findings in the field.

Some of these issues were addressed by Scholte *et al.*², whose research involved trials with cotton sheets impregnated with fungal spores in several dwellings in rural Tanzania. When such sheets are draped or hung in a house, mosquitoes will tend to rest on them — hence their designation as 'resting' sheets. The results confirm Blanford and colleagues' conclusions¹ that mosquito survival is significantly decreased by fungal infection. When Scholte *et al.* fed their data into an epidemiological model to calculate the effect on malaria transmission, the estimated number of infective mosquito bites per person per year dropped from 262 to 64. Increasing the coverage of mosquito resting sites could bring this number down to 10.

So far, so good. But what about the ecological and evolutionary issues that arise? The first is fungal specificity — or lack of it. Two of the isolates used by Blanford *et al.* came from beetles and moths, moth isolates also being used by Scholte and colleagues. The fungi are likely to kill pretty much any insect, and maybe other organisms, that come into contact with them. Although people would probably be better off without most insect species that get into houses, that may not be true for all of them. Lack of specificity might not be a problem, but it merits further research.

Second, there is the question of the possible development of resistance to the fungi. Use of



Figure 1 | Blood sucker. An *Anopheles* mosquito, of a species that transmits malaria, takes a meal.

insecticides against mosquitoes, or drugs such as chloroquine against *Plasmodium*, have both resulted in the advent of resistance to these chemical assaults. What might happen if fungi are deployed as control agents? Could they actually make matters worse?

Mosquitoes might evolve ways to prevent the fungus from entering their body, or limiting its growth if they do become infected. Such forms of resistance are possible, but it seems unlikely that they would intensify *Plasmodium* transmission or virulence. Another form of mosquito resistance might be behavioural. Malaria-transmitting mosquitoes feed on the blood of both humans (Fig. 1) and domestic animals, host preference being genetically determined, at least partially^{3,4}. The widespread indoor application of fungi could impose strong selection on mosquito host-preference or resting behaviour, because only mosquitoes feeding or resting indoors would be infected. A shift in host preference towards domestic animals would have a lasting benefit in terms of malaria transmission to humans (human malaria develops only in humans). It could result in increased transmission of disease among domestic animals, but it is perhaps preferable to deal with a problem of economics rather than one of public health.

Another — perhaps more worrying — prospect is that the rate of *Plasmodium* development would accelerate, enabling the parasite to produce its transmissible offspring before the mosquito host is killed by the fungi. This would be a serious outcome, for two reasons. First, it implies that the malaria 'generation time' would decrease, resulting in more malaria per unit of time. We don't really understand why malaria takes so long to become transmissible⁵, but the effect of the fungi could be to shift the balance towards faster development. Second, faster development could be associated with higher virulence in humans. The correlation of parasite traits in their different hosts is poorly understood⁶, and analyses of the variation of developmental time among *Plasmodium* isolates and its relation to virulence in mosquitoes and vertebrate hosts are called for. A reassuring result in that

respect is that, in the mouse model of malaria, *Plasmodium* virulence in the vertebrate and the mosquito are not correlated⁷.

Having raised various concerns, we should return to the promise of mosquito-killing fungi for malaria control. The fungi evidently have a strong effect on malaria transmission, and they target the transmitting stage of the mosquito, the blood-feeding adult. The approach is environmentally friendly, at least compared,

for example, with spraying larval insecticides on water surfaces. And it can be enhanced by relatively straightforward measures such as increasing the dosages of the fungal spores or the size of the resting sheets, and using both impregnated bednets and resting sheets. There is also the possibility of increasing the longevity of the spores, through fungal-breeding programmes. All in all, we have the prospect of opening a new front in the war on malaria. It is surely an approach worth pursuing. ■

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ENGINEERING

Skimming the surface

Jacob N. Israelachvili

Models of the microscopic contact area between two surfaces work surprisingly well, or fail completely, depending on the aspects of adhesion or friction being investigated. A simulation now shows how the details matter.

What happens at the atomic and molecular level when surfaces come into contact with each other? And how do these events relate to macroscopic properties and observations? These questions, which centre on the phenomena of adhesion and friction, pose challenges not only in engineering but in many other areas of the physical and biological sciences. Finding correlations and models that connect the atomic and macroscopic worlds is not easy. On page 929 of this issue, Luan and Robbins¹ describe the use of molecular dynamics to test the limits of macroscopic descriptions. The novel conclusions that they reach highlight just how important the atomic-scale details can be in controlling the behaviour of surfaces as they adhere to and slide past each other.

Macroscopic theories usually sidestep the atomic structure of matter, and instead view the interacting objects as smooth, with structureless surfaces. Such 'continuum' models of adhesion, a field known as contact or adhesion mechanics, are based on the pioneering theories of Hertz^{2,3} and of Johnson, Kendall

and Roberts (JKR)⁴. They use linear elasticity theory to describe the deformations of two smooth, curved surfaces when they are pressed together or separated through contact that is non-adhesive (Hertz theory) or adhesive (JKR theory). Adhesive contact means that the surfaces naturally stick to each other. In other words, the surface energy, generally denoted γ , is finite. In air, all surfaces have a finite γ , so they stick to each other provided the surfaces are atomically smooth over their entire macroscopic contact area. In liquids, surfaces — even smooth ones — can repel each other, leading to lubrication rather than friction forces.

JKR theory predicts a remarkably simple equation for the adhesion force F needed to detach a surface of radius R from a flat surface: $F = 3\pi R\gamma$ (Fig. 1, overleaf). Detachment occurs when the surfaces require a negative load in order to separate. The equation can be generalized to other geometries by replacing R with some characteristic length for that geometry. The JKR equation holds surprisingly well, but only for perfectly elastic bodies with atomically smooth surfaces and radii much larger than



50 YEARS AGO

The resemblance between the X-ray photographs of B8 and those of tobacco mosaic virus may perhaps appear rather slender. However, the strong similarity between B8 and tobacco mosaic virus protein in other respects (immunochemical cross-reactions, size of the basic chemical unit, and diameter of the rod-shaped particles) makes it very probable that a close structural relationship exists...

If we are right in thinking that the structure of B8 is closely related to that of tobacco mosaic virus protein, then considerable importance must be attached to the reversal of the sign of birefringence in B8. We must conclude that the ribonucleic acid makes a positive contribution to the birefringence of tobacco mosaic virus, and hence that the purine and pyrimidine rings are aligned approximately parallel to the axis of the particle. If the ribonucleic acid in tobacco mosaic virus forms a central core... it follows that its structure must differ considerably from the structure of deoxyribonucleic acid described by Crick and Watson.

Rosalind E. Franklin

From *Nature* 18 June 1955.

100 YEARS AGO

"The Inheritance of Acquired Characters." Is the following an instance of such inheritance? Lately I heard a missionary at a May meeting tell of the marvellous facility with which Chinese children memorise whole books of the Bible; the four Gospels, and sometimes the Acts also, being an easy feat for children of ten or twelve years. Having carefully sought information from other authorities, I find these facts confirmed, and that the same applies to Mohammedan children. We are aware that for ages their ancestors have been compelled to memorise long portions of their sacred books, and although occasionally we meet with a child of any nation with a gigantic memory, that differs widely from the case of a people where it has become a general characteristic.

From *Nature* 15 June 1905.

atomic dimensions — the sort of ideal characteristics for which the theory was intended.

Unfortunately, most bodies and surfaces are far from ideal, and both elastic (especially viscoelastic or plastic) properties and surface roughness can affect adhesion and friction. In the case of polymer surfaces, the adhesion force can be many orders of magnitude higher than the JKR value; for hard, rough surfaces, it can be many orders of magnitude lower. Hence the need for more refined theories. In addition, there is growing interest in contacts with nearly atomic dimensions, for example in microelectromechanical systems that often fail because of undesired adhesion.

Luan and Robbins¹ used molecular simulations to compare the atomic-scale behaviour of deformations, local stresses, adhesion and friction with that predicted by continuum theories. They considered three types of curved surface: a bent crystal lattice, or atomically smooth surface; an amorphous, randomly rough material with a curved, randomly rough surface; and a stepped surface whose steps are cut from a crystal lattice to produce a macroscopically curved surface (see Fig. 1 on page 929). All had the same average radius of curvature with root-mean-square deviations of less than an atomic diameter, yet the small differences in surface structure led to dramatic changes in behaviour.

A particularly important implication of this work is that the commonly used term 'surface roughness' can hide a multitude of effects. Surface bumps (asperities) can be regular (periodic, such as a sine wave) or irregular (random), and both can have a range of horizontal and vertical length scales. A perfect lattice is periodic, but so is a surface that has been nano- or micromachined to have a regular array of holes or channels. Two such periodic surfaces may be commensurate (when the hills and valleys on opposite surfaces match) or incommensurate (when they don't match). All of these effects, the authors found, can produce very different deformations and forces, even when the 'roughness', as defined in the conventional way in terms of the root-mean-square amplitude (Fig. 1), is the same.

Luan and Robbins observed that, for atomically smooth surfaces, the Hertz and JKR theories work well when describing the macroscopic contact area as a function of load, and the stress distribution within that contact. But both the rough and stepped surfaces showed large fluctuations in the local pressures or stresses and greatly reduced adhesion forces F compared with the JKR prediction. The stepped surface had the largest deviations from continuum behaviour, showing sharp discontinuities in the contact area with load. The friction forces were even more sensitive to surface structure, as previously found experimentally⁵, with rough and incommensurate surfaces having very low friction.

These results¹ have both fundamental and practical implications. On the fundamental

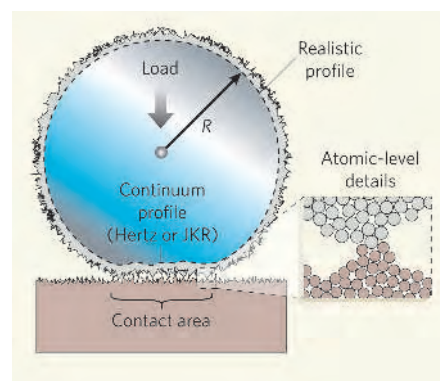


Figure 1 | Forms of mechanical contact.

Continuum theories (dashed black circle of radius R) predict the average macroscopic profiles of adhesive (JKR) or non-adhesive (hertzian) contacts when two surfaces are pressed together by a load. In their study, Luan and Robbins¹ also use a realistic profile that preserves the underlying atomic-scale surface structure of the material. This inherent roughness (represented by a root-mean-square roughness) determines the macroscopic adhesion and friction forces.

side, they show that mean field theories, in which properties such as surface roughness or texture are averaged or smeared out, are doomed to grossly oversimplify the real situation. On the practical side, they show how surfaces might be tailored to interact in desirable ways, but only if the atomic-scale details are taken into consideration. In this regard, the results bear out what has long been known in biology: fine details often determine macroscopic behaviour. For example, the precise primary sequence of amino acids determines protein folding and so a protein's properties.

There is much still to do before the physical interactions between 'real' surfaces are fully understood. First, the asperities on most surfaces have a complex hierarchy of length scales from the atomic to the macroscopic; Luan and Robbins considered only a narrow class of these, namely atomic-scale asperities. The relative roles of stiffness and adhesion are believed to depend on the detailed shapes of surface bumps. Second, they considered only the limit at which stiffness dominates over adhesion, so that deformations remained relatively small, as for hard crystalline surfaces such as metals and ceramics. In contrast, soft polymeric and biological surfaces often deform into full contact by adhesive forces. And finally, non-equilibrium and rate-dependent effects often determine how real surfaces interact in 'real time'. These interactions include rate-dependent friction and lubrication forces. The results of Luan and Robbins are illuminating — they have just skimmed the surface of this complex class of phenomena, but indicate just how rich the behaviour can be.

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CELL BIOLOGY

Powerful curves

L. Mahadevan and T. J. Mitchison

A cell's contents are organized by a scaffolding of microtubules. These long, thin polymers continuously grow and shrink, and the structures of two forms of the constituent protein provide clues to how this occurs.

Microtubules are long polymers of the protein tubulin that form a network within cells to help arrange the cell components and provide transport tracks for motor proteins. Rather than being static permanent structures, microtubules continuously grow and shrink through the polymerization and depolymerization of tubulin. Such processes are central to the microtubules' spatial organization and their ability to generate the forces necessary to function. In this issue, Wang and Nogales (page 911)¹ report high-resolution structures of two alternative polymeric states of tubulin, which provide insights into the molecular mechanisms that power growth and shrinkage.

Tubulin is a stable dimer of α and β subunits, both of which bind guanine nucleotides. Guanosine triphosphate (GTP) that is bound to β -tubulin is hydrolysed to guanosine diphosphate (GDP) during microtubule assembly, and this nucleotide regulates tubulin conformation and behaviour, with GTP favouring polymerization, and GDP depolymerization. In the presence of tubulin and GTP, individual microtubule ends tend to grow for many micrometres, and then switch to shortening. This transition, called a catastrophe, occurs spontaneously with pure tubulin and constant GTP levels, although in cells it is regulated by other proteins. The resulting 'dynamic instability'² allows microtubule ends to efficiently explore their surroundings³ and to perform mechanical work by pushing and pulling⁴. A central question is how the chemical energy from GTP hydrolysis is harnessed to power both growth and shrinkage of microtubules in dynamic instability.

Initial models emphasized a thermodynamic–kinetic view. GTP-bound tubulin subunits have a high affinity for microtubule ends and dissociate slowly, whereas GDP-bound tubulin subunits have a low affinity and dissociate quickly². A proposed kinetic lag between polymerization and hydrolysis could generate a 'GTP cap' that stabilizes growing ends. Definitive evidence for or against such a cap is still lacking.

More recently, cryo-electron microscopy of growing and shrinking microtubules⁵ suggested a complementary structural–mechanical view,

based on changes in the arrangement of tubulin subunits in the polymer lattice. Tubulin molecules in microtubules are arranged in 13 lines called protofilaments, which lie parallel to the microtubule axis. When microtubules depolymerize, these protofilaments curve outwards, and in the presence of microtubule-associated proteins or certain divalent cations, they bend back on themselves to form stable rings of GDP-tubulin (Fig. 1a)⁶. GTP hydrolysis was proposed to destabilize microtubules, and drive dynamic instability, by promoting outward curving⁵, although the mechanism coupling hydrolysis to curving was unknown.

By comparing the structure of GDP-protofilament rings¹ with that of microtubules⁷, Wang and Nogales¹ reveal how GTP hydrolysis promotes protofilament curving and thus destabilizes the microtubule lattice. The GDP-protofilament is bent at both the inter- and intra-dimer interfaces, making it curve outwards from the microtubule. Within

the main microtubule, most subunits are bound to GDP, and thus their lowest energy state would be this curved form. However, contact with neighbours in the lattice forces the protofilament to be straight, except at the ends. In this way, the microtubule lattice captures chemical energy from GTP hydrolysis and stores it in the form of mechanical strain energy. Depolymerization releases this strain energy, making the reaction energetically favourable, even in the presence of high concentrations of GTP-tubulin.

Wang and Nogales¹ also solved the structure of tubulin with GMPCPP, an analogue of GTP, bound to β -tubulin. This analogue is not hydrolysed during polymerization, and by mimicking GTP it locks tubulin in the GTP conformation. Simple dynamic instability theory predicts that the preferred conformation of GTP-tubulin should be that of the microtubule lattice; that is, straight protofilaments. But in the GMPCPP structure they in fact curve outwards, albeit to a lesser extent than GDP protofilaments. This structure required cooling, which induces a conformational change in tubulin, so the geometry might differ from anything that occurs normally. With that caveat, the combined data support a two-step model for microtubule growth, with initial polymerization into gently curved sheets, followed by tube closure^{1,5}. Exactly when GTP hydrolysis occurs is not clear. GTP analogue protofilaments roll up into microtubules on warming¹, showing that hydrolysis is not necessary for tube closure. The alternative forms of the GTP-tubulin lattice probably have similar energies, and may interconvert at growing ends, while maintaining a GTP cap (Fig. 1a).

A greater understanding of how tubulin

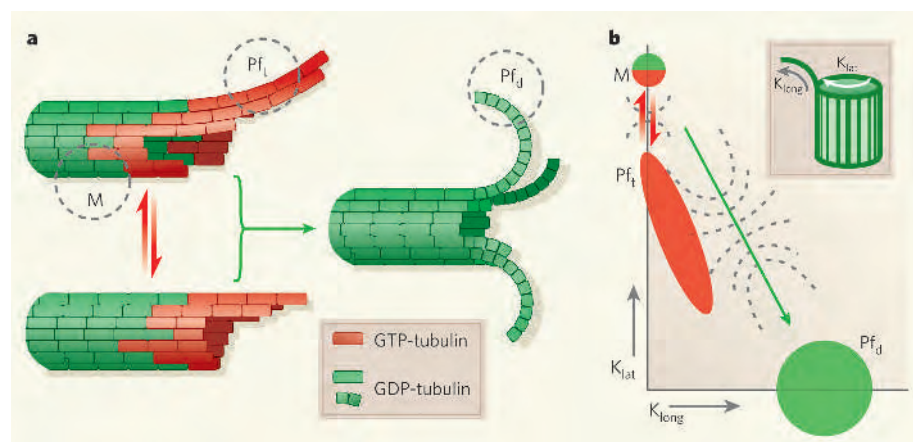


Figure 1 | Dynamic microtubule structure. **a**, A synthesis of the thermodynamic, kinetic and structural views showing the growing and shrinking microtubule ends. Growing ends (left) fluctuate between gently curved and straight protofilament sheets; shrinking ends (right) are dominated by highly curved, peeling protofilaments. Structures have been solved for three forms of the microtubule lattice: microtubule (M)⁷, GTP-protofilament (Pf_t)¹ and GDP-protofilament (Pf_d)¹. **b**, Our model of a free-energy landscape for the microtubule lattice. Each of the three metastable forms of tubulin polymer can be approximately specified by two curvatures: K_{long} parallel to and K_{lat} perpendicular to the microtubule axis (inset). Coloured shapes represent these forms, corresponding to low-energy wells in the landscape. The larger wells are less geometrically constrained. Dotted lines represent energy barriers. GTP-tubulin (red) interconverts rapidly between M and Pf_t forms across a low barrier. GDP-tubulin (green) crosses the higher barrier between M and Pf_d less frequently, and perhaps irreversibly.

structure and thermodynamics together drive dynamic instability requires modelling. Lattice simulation using molecular dynamics has yielded promising results⁸, and the new structures will help to refine such detailed models. A simpler approach that we propose, taking the new findings into account, is to approximate the lattice as an elastic sheet with more than one equilibrium configuration, and with the curvature of the sheet providing a natural description of the geometry (Fig. 1b, inset). Naturally curved elastic sheets can be bistable, interconverting between two forms, where curvature in one direction is partially exchanged for curvature in a perpendicular direction⁹. These forms may have approximately equal free energy, but with a barrier separating them, because interconversion requires local stretching of a small region that must propagate along the sheet.

We have generated a possible energy landscape for GTP-tubulin and GDP-tubulin lattices based on this geometric view (Fig. 1b). For GTP-tubulin, the microtubule (straight) and protofilament (gently curved) forms have similar energies that are separated by a relatively low barrier. They interconvert at the growing microtubule end, crossing the barrier by exchange of curvatures. The energy of the GDP-tubulin protofilament (highly curved) form is lower than that of all other forms. Catastrophes occur when ends occasionally cross the higher barrier separating the straighter microtubule and GTP-protofilament forms from the highly curved GDP-protofilament form. The height of the barrier is determined by the energetic cost of exchanging curvature combined with tearing between protofilaments. Thus, rapid depolymerization is driven by the elastic energy stored in straight GDP-protofilaments as they recover their natural curved shape, after a tear travels down from the free end of the microtubule. In this type of model, the relative stiffness and strength of the intra- and inter-protofilament bonds¹⁰ is crucial in determining the rate of catastrophes.

Our model makes qualitative predictions: microtubules with fewer protofilaments and smaller radii should have higher energies for curvature exchange, and so are likely to grow more slowly and undergo catastrophe less frequently, because both processes require curvature exchange. Once they suffer a catastrophe, however, they should shrink faster, because the density of stored elastic energy is greater. Microtubule radius can vary, which might explain the variation in polymerization dynamics of individual microtubules assembled from pure tubulin¹¹.

The structural complexity of growing microtubule ends may have important consequences in cells. Tip-tracking proteins associate with growing plus-ends, where they regulate polymerization dynamics by unknown mechanisms¹². Perhaps such proteins target growing ends by binding to protofilament sheets, and regulate dynamics

by influencing tube closure. Investigating how tubulin lattice conformation influences tip-tracking proteins, and vice versa, will probably reveal some interesting biology.

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NUCLEAR PHYSICS

Elusive magic numbers

Robert V. F. Janssens

Gaps in nuclear levels, which cause nuclei with 'magic' numbers of protons or neutrons to be especially stable, seem to be different for nuclei with an excess of neutrons. But are all magic numbers aberrant in exotic species?

The idea of a shell structure is often cited by physicists as an essential aid to understanding the atomic nucleus. But the exact number of protons or neutrons required to fill a particular nuclear shell has not yet been conclusively settled. In a study of the neutron-rich silicon nucleus ⁴²Si, Fridmann and colleagues¹ (page 922 of this issue) provide an important contribution to the discussion.

The concept of shell structure is familiar

from atomic theory: the energy needed to remove the last electron from an atom varies with atomic number. Certain elements — those with a full outer shell of electrons — are more tightly bound than others, and are thus particularly stable chemically, not readily bonding or forming molecules with other atoms. These are the noble gases: helium, neon, argon, krypton, xenon and radon, with a total of 2, 10, 18, 36, 54 and 86 electrons, respectively.

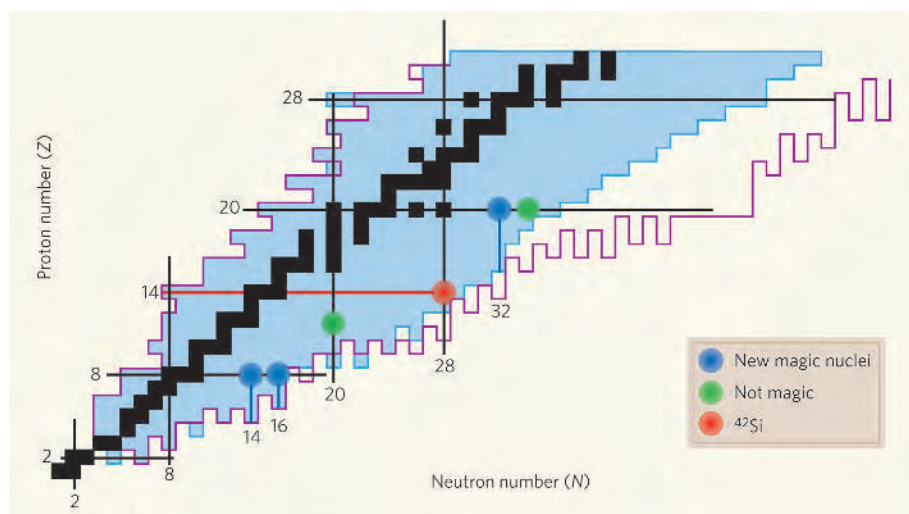


Figure 1 | Nuclear landscape. The stable elements from hydrogen (proton number $Z = 1$) to zinc ($Z = 30$) are represented by black squares; all other known bound nuclei are contained in the light blue area. At the 'drip lines' (violet lines), the forces between neutrons and protons are no longer sufficiently strong to hold nuclei together. The vertical and horizontal black lines indicate the magic numbers 2, 8, 20 and 28 that apply to stable nuclei. Some anticipated 'magic' nuclei are, in fact, not magic (green dots): the beryllium isotope ¹²Be ($Z = 4$, neutron number $N = 8$) and the exotic magnesium nucleus ³²Mg ($Z = 12$, $N = 20$) are examples. Besides the doubly magic standard oxygen isotope, ¹⁶O ($Z = N = 8$), the oxygen isotope with 20 neutrons, ²⁸O, should be particularly stable — but experiments show that it is not even bound. Conversely, there are strong indications of new magic numbers at $N = 14$, 16 and 32 in neutron-rich nuclei (dark blue dots). The silicon isotope ⁴²Si, the main subject of the work of Fridmann and colleagues¹, is marked by a red dot.

The energy required to remove the last proton or neutron from the atomic nucleus exhibits similar discontinuities as a function of both the number of protons, Z , and the number of neutrons, N , in the nucleus. Naturally occurring nuclei with proton or neutron numbers of 2, 8, 20, 28, 50 or 82 have enhanced stability. For neutrons, 126 is also such a magic number — the lead isotope ^{208}Pb (82 protons, 126 neutrons) is an example. The nuclear shell model², for which the 1963 Nobel Prize in Physics was awarded, describes protons and neutrons as occupying well-defined levels in a nuclear potential; magic numbers appear when large gaps in energy occur between two of these shells.

Studies during the past decade or so have shown that magic numbers are not as immutable as was once thought. So far, work has focused mainly, but not exclusively³, on light nuclei with proton and neutron numbers that are greatly different from those of stable nuclei. Some nuclei that were expected to be magic turn out not to be particularly tightly bound (Fig. 1). On the other hand, there are indications of new magic numbers for 'exotic' nuclei that have many more neutrons than protons (Fig. 1). These experimental observations suggest that the gaps in the sequence of nuclear levels that give rise to magic numbers depend on the balance between protons and neutrons in ways requiring further exploration. But are magic numbers different in all exotic species? Fridmann and colleagues' investigations¹ of ^{42}Si show that they are not.

In nature, there are three stable isotopes of silicon ($Z = 14$), which have 14, 15 and 16 neutrons, respectively. With 28 neutrons, ^{42}Si has 12 more than the heaviest stable isotope; producing and investigating such an extremely imbalanced nucleus has become possible only recently. Fridmann and colleagues accelerated ions of neutron-rich calcium (^{48}Ca) into a beryllium target, selecting sulphur (^{34}S) nuclei from the resulting fragments using a fragment separator⁴ and directing these on to another beryllium target. The second step can cause a 'two-proton knockout' reaction, producing ^{42}Si nuclei that can be identified in a magnetic spectrograph⁵. This reaction produces silicon in its lowest energy (ground) state, but can also produce it in states of higher energy that decay almost instantly to the ground state by means of γ -ray emission. An array of 18 γ -ray detectors around the target allowed this process to be detected.

Two-proton knockout was only one of many reactions that took place in the experiment, another being the creation of phosphorus nuclei (^{31}P) from the sulphur nuclei through the removal of a single proton. Fridmann and colleagues¹ gathered valuable, complementary information from studying the excitation of ^{43}P .

The probability of two-proton knockout from ^{44}S is, in fact, very small. By comparing the experimental data with calculations using

the 'eikonal' theory⁶, the authors show that this can be understood only if ^{42}Si does indeed have a magic character. As eikonal theory has not been tested extensively for projectiles with mass larger than 40, Fridmann and colleagues also measured, as an experimental control, the two-proton knockout from ^{46}Ar (another fragment of the primary ^{48}Ca beam) into ^{44}S , which has a well-known structure.

The studies of ^{42}Si show that the magic number $N = 28$ remains valid despite the large excess of neutrons in this nucleus. But a full understanding of the experimental observations requires that proton number $Z = 14$ be magic as well (previously, only $N = 14$ had been shown to be magic in exotic nuclei). This makes the silicon isotope doubly magic, and results in an almost spherical shape for the nucleus.

Such results indicate that some aspects of the interactions responsible for shell structure in nuclei are not readily apparent from the properties of the stable nuclei; rather, these aspects are amplified in exotic systems located far from the stable ones. To provide a satisfactory description of all nuclei, the challenge remains to understand what mechanisms cause changes in nuclear shell structure as the numbers of protons and neutrons change.

If these aspects of the forces that bind nuclei are less important in stable nuclei, why worry?

The reason is that nature does not deal solely in stable nuclei: processes such as the nuclear reactions in stars often involve far-from-stable exotic nuclei, especially the nucleosynthetic processes in stellar explosions that produce nuclei heavier than carbon and oxygen. Reaction timescales under stellar conditions are frequently so short that, once an unstable nucleus is produced, it gets involved in a nuclear reaction before it can decay. An understanding of how the elements were, and continue to be, made in the Universe depends on our ability to calculate the reaction rates for their production. These rates in turn depend critically on the shell structure of exotic nuclei — precisely where the presence or absence of a magic number has a considerable impact. ■

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DEVELOPMENTAL BIOLOGY

One source for muscle

Iain W. McKinnell and Michael A. Rudnicki

Producing muscle as an embryo, and making or repairing it as an adult, could be considered to be quite different processes. But it seems that cells that share a common origin carry out both of these tasks.

The generation and repair of skeletal muscle is a highly ordered, multi-step process that requires many progenitor cells and continues throughout embryonic, fetal and postnatal life. In this issue, Gros *et al.* (page 954)¹ and Relaix *et al.* (page 948)² show that a common progenitor cell not only maintains muscle growth during late embryonic development, but also seems to be the origin of the satellite cells responsible for postnatal muscle growth and repair.

The cellular origin of skeletal muscle is the somites — segmented blocks of tissue that form on either side of the developing spinal cord in the embryo (Fig. 1a). But the exact mechanism by which somitic cells generate the total skeletal-muscle mass is poorly understood. During early embryonic development the somites, resembling a tightly clustered ball of epithelial cells, undergo a programme of maturation and specialization ('differentiation') to produce the sclerotome, which forms

the skeleton, and the dermomyotome, which will form both the skin (dermis) and skeletal muscle (Fig. 1b).

This initial generation of muscle relies on proliferating muscle precursor cells from the borders of the dermomyotome, which ingress beneath the dermomyotome to form what we should now refer to as the primary myotome, a sheet of non-proliferating muscle-precursor cells called myoblasts (Fig. 1c)³. Notably, the dermomyotome is a transient structure that can produce only a limited number of myoblasts. It cannot support the progressive and extensive growth of muscle throughout embryogenesis, and so how the total muscle mass is generated remains a puzzle.

During later stages of embryogenesis, cells termed satellite cells are observed. These cells form a self-replenishing pool of muscle-specific stem cells responsible for postnatal muscle growth and repair. Their embryonic origin is unclear, as traditional embryological studies

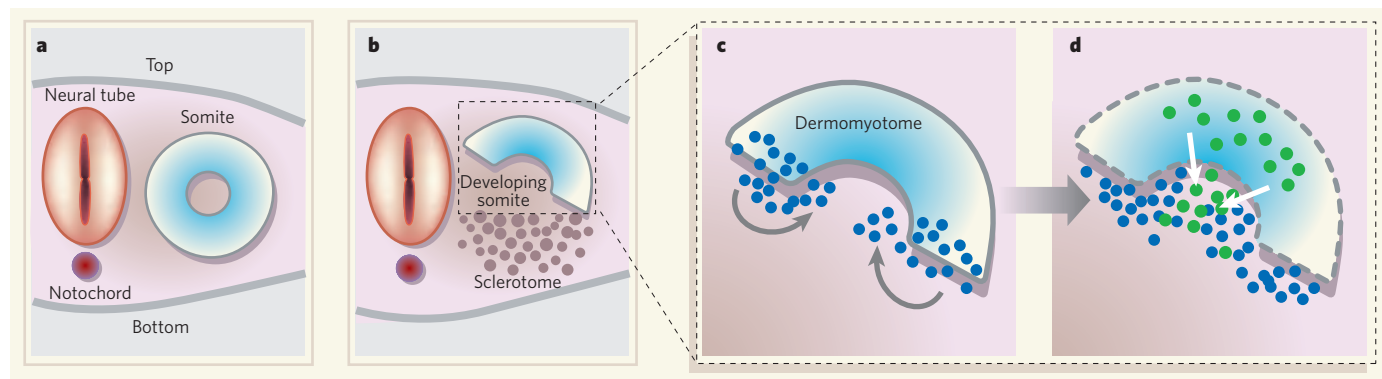


Figure 1 | The making of muscle. **a**, Somites form on either side of the developing spinal cord (notochord) of the embryo. One side is shown in cross-section. **b**, The dermomyotome, which will give rise to muscle and skin, develops from the somite. **c**, Proliferating muscle precursor cells ingress from the borders of the dermomyotome to form a sheet of non-proliferating, differentiating myoblasts — the primary myotome. **d**, Pax3- and Pax7-expressing cells originate from the central domain of the dermomyotome^{1,2}. They migrate into the primary myotome, proliferate and do not express markers of myogenic differentiation.

seem to indicate a somitic origin⁴, although more recent evidence implies they might be derived from the embryonic vasculature^{5,6}.

Gros *et al.*¹ and Relaix *et al.*² have used different cell-labelling strategies to track the movement and persistence of a population of previously undefined skeletal-muscle progenitor cells that originate from the central domain of the dermomyotome. Following cell division, one daughter cell migrates into the primary myotome and continues to proliferate (Fig. 1d), without expressing proteins that signal differentiation into specialized muscle cells. This is contrary to previous theories that the myotome cells become specialized and do not divide — the newly defined cells proliferate in the myotome to produce a source of skeletal muscle.

The authors found that, unlike the cells that initially form the myotome, the skeletal-muscle progenitor cells do not ingress from the borders of the dermomyotome. Instead, they move directly from the central dermomyotome region as it undergoes a transition from epithelial to mesenchymal cells, a process that signals the onset of dermis formation⁷ and the disintegration of the dermomyotome. Interestingly, around 90% of the skeletal-muscle progenitor cells express both the gene for Pax7, a distinct molecular marker of satellite cells, and that for Pax3, which is implicated in determining the fate of muscle cells. A smaller proportion (less than 10%) express either one gene or the other. The Pax3- and Pax7-expressing cells persist throughout the later stages of development, and go on to form skeletal muscle. In embryos in which both Pax3 and Pax7 are mutated, the cells that would normally coexpress Pax3 and Pax7 adopt non-muscular fates, such as bone or cartilage².

The satellite cells that emerge during late embryonic development account for the vast majority of muscle progenitor cells. Significantly, the authors of both papers observed that the skeletal-muscle progenitor cells from the central dermomyotome move to where satellite cells reside. Given that these cells accounted for more than 90% of cells in this location, this strongly suggests that most, if not

all, satellite cells are derived from the central dermomyotome of the somite. The demonstration that muscle progenitor cells can contribute to the satellite-cell population need not exclude a contribution from other sources; however, it could be argued that any alternative source will be less significant.

Further characterization of these novel skeletal-muscle progenitor cells should be informative. In particular, it would be useful to know when they coexpress Pax3 and Pax7 as opposed to expressing either gene alone, and the effect this has on their fate — do they form skeletal muscle immediately or become satellite cells? Given that only a few cells that are mutated in both Pax3 and Pax7 adopt non-muscle fates, these muscle-progenitor cells may well represent a mixed population of cells with differing developmental potential. That said, these studies^{1,2} are highly significant because they describe both the second stage of

myotome formation (vital for the generation of muscle precursors required for continued muscle growth) and a developmental route for the origin of satellite cells. Remarkably, both sets of progenitors share the same cellular origin — the hitherto unappreciated central portion of the dermomyotome.

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CELL BIOLOGY

New cog for a familiar machine

Mary Dasso

During cell division, intricate cellular machinery separates duplicated DNA into daughter cells. Unexpectedly, the assembly of this crucial apparatus seems to rely on components other than proteins and DNA.

When a cell divides, in a process known as mitosis, the duplicated genetic material must be divided and distributed precisely between the two 'daughter' cells. This task is the responsibility of an elaborate structure called the mitotic spindle, which forms during mitosis¹. The spindle contains two antiparallel arrays of dynamic microtubules. The 'minus' ends of these arrays are organized into two poles around microtubule-nucleating organelles called centrosomes, and their 'plus' ends extend towards the chromosomes that are aligned in the middle of the spindle. Apart from the DNA of the chromosomes them-

selves, the spindle was thought to consist solely of proteins²; but another factor vital for its formation has come to light. Blower and colleagues³, writing in *Cell*, find that RNA is essential for spindle assembly.

The formation of the spindle during mitosis requires two proteins³, called Ran and Importin- β . When cells are not in mitosis, these proteins help to control molecular traffic between the cytoplasm and the nucleus. Importin- β binds to specific proteins in the cytoplasm that are destined for the nucleus, and mediates their passage across the nuclear envelope. Ran binds to guanosine

diphosphate (GDP) or guanosine triphosphate (GTP), and the switch from GDP to GTP is catalysed in the nucleus by an 'exchange factor' called RCC1. Ran-GTP interacts with Importin- β (Fig. 1), causing it to release its import cargo because binding of Importin- β to its cargo and to Ran-GTP are mutually exclusive.

In mitosis, however, the nuclear envelope of vertebrate cells breaks down, and during this time, Importin- β binds and inhibits a variety of 'spindle assembly factors'³. As before, Ran-GTP can affect these interactions. It is widely believed that RCC1 binds to chromosomes during mitosis and increases the local concentration of Ran-GTP, thereby activating spindle assembly factors near the chromosomes to guide the spatial organization of the spindle. As a result of its capacity to activate spindle assembly factors, the addition of Ran-GTP to extracts from eggs of *Xenopus laevis* frogs (a popular model system for studying spindle formation) is sufficient in itself to cause the formation of spindle-related microtubule structures called asters in the absence of DNA.

Blower *et al.*² used an activity-based assay to purify spindle assembly factors that bind to Importin- β in *Xenopus* egg extracts. They isolated a protein called Rae1. Rae1 was previously shown to be essential for exporting messenger RNAs (mRNAs) from the nucleus in yeast⁴, although its precise role is poorly understood. Rae1 localizes to the spindle poles and to chromosomes in egg extracts², and its depletion abolished both spindle assembly around sperm DNA and the formation of asters in response to Ran-GTP in DNA-free extracts.

Further analysis of the egg extracts showed that Rae1 associates with a number of proteins that are parts of large RNA-protein complexes called ribonucleoproteins (RNPs)². These RNP components included the Maskin protein, which is recruited to mRNAs to control translation of the RNA code into protein during early *Xenopus* development. To test whether the integrity of the RNPs affects Rae1 function, Blower *et al.* treated egg extracts with an enzyme that destroys RNA (RNase). Remarkably, they found that this treatment disrupted spindle assembly around chromosomes and aster formation in response to Ran-GTP.

Treatment with protein-synthesis inhibitors did not affect spindle formation under the same conditions. The authors therefore argue that the effect they observed is not due to the translation of the mRNAs. However, it should be noted that other researchers have found that protein synthesis may be important later in the process of spindle assembly⁵. Blower and colleagues' findings for the first time implicate RNA as an essential component of the spindle, and imply that RNPs have a structural or regulatory role during early steps in spindle formation that can be distinguished from their role in protein synthesis.

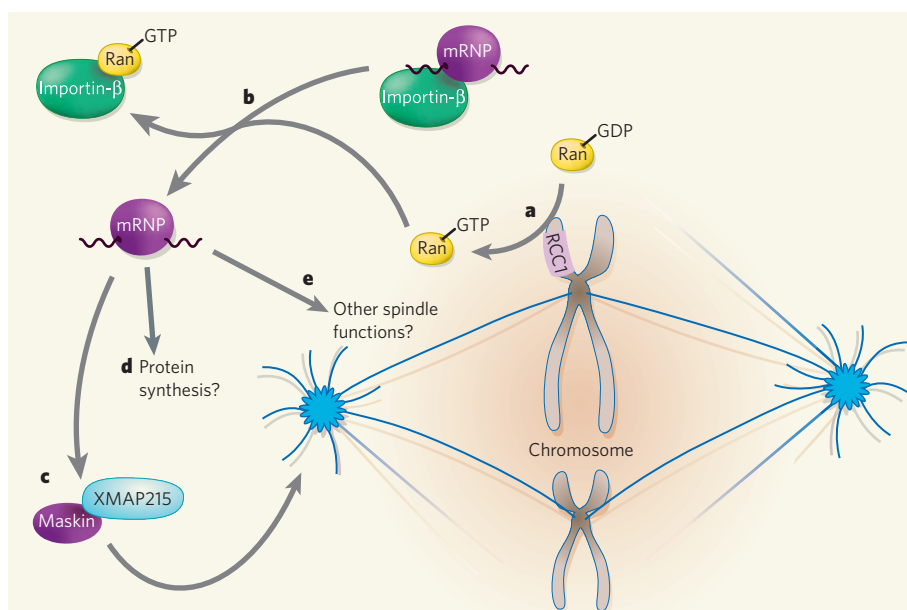


Figure 1 | RNPs and spindle assembly. **a**, Ran-GTP is generated by the chromosome-associated exchange factor, RCC1. **b**, Blower *et al.*² find that Ran-GTP releases the direct binding of Importin- β to Rae1-associated messenger RNA-protein complexes (mRNPs). **c–e**, RNPs such as Maskin may have many functions during cell division, including targeting XMAP215 to the spindle poles³ (**c**), regulation of protein synthesis (**d**) and other structural or regulatory roles in spindle assembly (**e**).

A major question is how RNPs function in spindle assembly. One possible mechanism is suggested by another recent study by O'Brien *et al.*⁵, who investigated the role of Maskin in spindle assembly. Maskin is a member of the 'transforming acidic coiled coil' (TACC) protein family, and is the first TACC protein to have been found in *Xenopus*⁶. In mammals and flies, TACC proteins reside at centrosomes, and interact with the XMAP215 and Aurora A proteins^{6,7}. XMAP215 is a microtubule-associated protein that modulates microtubule dynamics⁸, and Aurora A controls its activity⁷. Aurora A is also a downstream target of Ran, through an Importin- α/β -regulated spindle assembly factor called TPX2 (ref. 7).

O'Brien *et al.*⁵ found that Maskin interacts with XMAP215 in a manner that seems similar to how other TACC proteins act: although XMAP215 can bind to microtubules in Maskin-depleted egg extracts, it fails to accumulate at spindle poles. The Maskin-depleted extracts form undersized asters in response to Ran-GTP and assemble highly disorganized spindles around added sperm chromosomes. These findings show that Maskin's control of XMAP215, and perhaps of other spindle components, is essential for proper spindle assembly. Notably, RNase treatment, Maskin depletion and Rae1 depletion give related but distinct outcomes, possibly suggesting that many different RNPs have diverse roles in spindle assembly^{2,5}.

Collectively, these data hint at a complex web of interactions at spindle poles that are carefully regulated to achieve balanced microtubule assembly and spindle organization. Naturally, many questions remain to be addressed, including how Ran regulates these

interactions at such relatively large distances from RCC1 activity on mitotic chromosomes, whether the incorporation of RNPs in the spindle might modulate the translation of individual mRNAs or their distribution to daughter cells, and whether these RNA-based mechanisms also occur in non-embryonic systems and among different species. ■

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CORRECTIONS

● In the News & Views article "Plant biology: Auxin action" by Judy Callis (*Nature* **435**, 436–437; 2005), editorial error introduced the implication that auxins are proteins. They are not. Naturally occurring auxins are low-molecular-weight compounds derived from the amino-acid tryptophan or a tryptophan precursor.

● In "Organic chemistry: Fast reactions 'on water'" by Jaap E. Klijn and Jan B. F. N. Engberts (*Nature* **435**, 746–747; 2005), the page numbers for the main paper under discussion (ref. 2) were wrong in the print edition. The correct reference, which appears in the online edition, is Narayan, S. *et al.* *Angew. Chem. Int. Edn* **44**, 3275–3279 (2005).

BRIEF COMMUNICATIONS

Relatedness among east African coelacanths

Scattered groups of these ancient fish may all stem from a single remote population.

Coelacanths were discovered in the Comoros archipelago to the north-west of Madagascar in 1952. Since then, these rare, ancient fish have been found to the south off Mozambique, Madagascar and South Africa, and to the north off Kenya and Tanzania — but it was unclear whether these are separate populations or even subspecies. Here we show that the genetic variation between individuals from these different locations is unexpectedly low. Combined with earlier results from submersible and oceanographic observations^{1,2}, our findings indicate that a separate African metapopulation is unlikely to have existed and that locations distant from the Comoros were probably inhabited relatively recently by either dead-end drifters or founders that originated in the Comoros.

To estimate genetic variation, we analysed mitochondrial DNA sequences and microsatellite DNA from a total of 47 coelacanths (*Latimeria chalumnae*) from all of the different African locations apart from Tanzania (Fig. 1, red circles). (For methods, see supplementary information.) No differences were found in the mitochondrial sequences encoding cytochrome *b*, but six different haplotypes could be defined by nucleotide variations at various positions in the control region (for details, see supplementary information).

The three haplotypes not found in fish from the Comoros differ by only a single nucleotide from the most frequent Comoran haplotype, from which we infer that fish from all locations tested are closely related. (For comparison, we found a difference of 7.0–7.3% in the mitochondrial control-region nucleotide sequences of *L. chalumnae* and the Indonesian coelacanth *L. menadoensis*³.)

We also typed ten informative microsatellite loci using a total of 38 alleles (see supplementary information). No allele was restricted to fish from any one of the locations outside the Comoros. The relatedness between animals from the Comoros also extended to those from Mozambique, Madagascar, Kenya and South Africa. The genetic homogeneity indicated by this microsatellite analysis agrees with results from multilocus fingerprinting of samples from Mozambique and the Comoros⁴. Individual heterozygosity was low, which

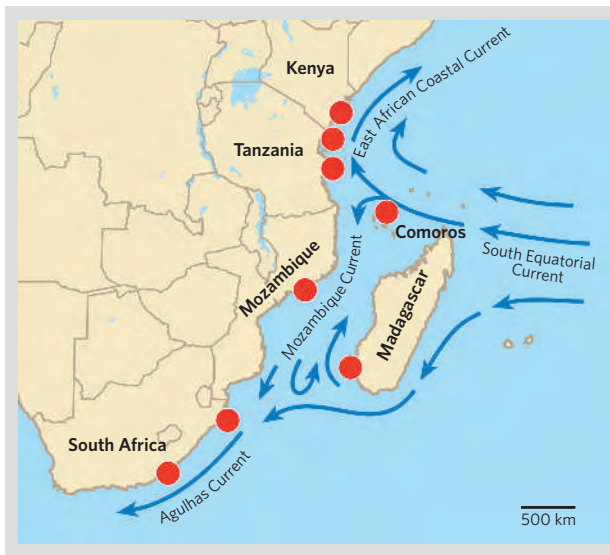


Figure 1 | Where the coelacanths are. Principal ocean currents and recorded coelacanth localities (red circles) in the western Indian Ocean.

is generally taken as a sign of inbreeding⁵.

There were therefore no significant genetic differences in any of the coelacanths tested. Our results indicate that either all the specimens belong to a single, large, interbreeding (panmictic) population, or that there was a recent subdivision that could not be detected in our analysis. The shared mitochondrial haplotypes and microsatellite alleles in coelacanths from the Comoros and from other locations could be the result of free gene flow; however, the South Equatorial Current divides near the Comoros into the prevailing southerly Mozambique Current, which is dominated by a train of large anticyclonic eddies², and into the northerly East African Coastal Current (Fig. 1), which might prevent gene flow from the south or the north to the Comoros.

Coelacanths remain at their home sites for many years^{1,6}. On the basis of our observations from a submersible and our acoustic tracking experiments⁶, we believe that a regular benthic or pelagic migration across the deep ocean basins between the Comoros and the African or Madagascan mainland is highly unlikely, so a panmictic African population probably does not exist.

All coelacanths living outside the Comoros may have originated in the Comoros and/or other, as yet unknown localities in the Indian Ocean; they could be new arrivals, dead-end drifters (strays) or founders of young, repro-

ducing subpopulations. Of 24 coelacanths we identified during surveys from our submersible off South Africa, two were gravid females, representative of a small founder population. And a female carrying developed eggs with visible embryos was also recorded among at least 19 coelacanths caught off Tanzania⁷.

It is possible that the western Indian Ocean could have been colonized relatively recently by coelacanth drifters from the Pacific province: this sluggish fish might be expected to survive long, passive oceanic transport⁸ by the Indonesian throughflow, which has probably existed for only about 3 million to 4 million years (ref. 9), into the South Equatorial Current that flows to the west. This idea is supported by molecular-clock evidence (our unpublished results) and by the low

genetic diversity found in the Comoran and African populations.

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FOOD-WEB TOPOLOGY

Universal scaling in food-web structure?

Arising from: D. Garlaschelli, D. Caldarelli & L. Pietronero *Nature* **423**, 165–168 (2003)

The statistical analysis of empirical food webs seeks to discover patterns in their structure. Garlaschelli *et al.*¹ describe food webs as transportation networks and show that the empirical webs used in their study have universal scaling exponents. Here we analyse 17 of the most comprehensive food webs — including the nine used by the authors¹ — but find no evidence for this universality. We also argue that the exponents that are observed are not a signature of food-web architecture but are a general property of networks that have few trophic levels, irrespective of their structure. We conclude that the short range of empirical exponents occurs because food webs contain only a few trophic levels and therefore that it does not add to our understanding of food-web topology.

For each empirical food web, Garlaschelli and colleagues build several spanning trees and, for every species *i*, compute the number *A_i* of species that directly or indirectly feed on *i* (plus itself), and the ‘cost’ function *C_i* = ∑_{*k*} *A_k*, where the sum extends over the same set as for *A_i*. The authors report scaling relations *C_i* ∝ *A_i*^η, where the exponent η is in the range 1.13–1.16, and interpret this result as a universal property of food-web topology and a proof of self-similarity.

However, our analysis of plots of *C_i* against *A_i* for 17 food webs (Fig. 1) yields η values ranging from 1.09 to 1.26 (Table 1). The error is about 0.03 in all cases (95% confidence). The observed exponents therefore span a much larger range than the error and do not display universality, which would require the same value. The discrepancies observed between Table 1 and the values reported by Garlaschelli *et al.* are probably due to errors in their treatment of the raw data, which we have shown for the grassland empirical food web. Unlike Garlaschelli *et al.*¹, we used manipulated data sets supplied by N. D. Martínez’s group that have been verified and used in their publications.

Table 1 | Exponents for empirical food webs

Name	η	Name	η
Little Rock	1.11	Canton	1.09
Ythan 91	1.11	El Verde	1.09
Ythan 96	1.12	Reef*	1.10
Coachella	1.14	Stony	1.10
Silwood	1.15	Shelf†	1.17
St Marks	1.18	Bridge Brook	1.20
St Martin	1.19	Benguela‡	1.20
Skipwith	1.22	Chesapeake	1.25
Grassland	1.26		

Details of the food webs are cited in ref. 5, except the *Caribbean Reef², †northeast US Shelf⁴ and ‡Benguela³.

Empirical food webs mostly have three levels and, when present, the number of species in the fourth level is very small^{2–5} (the most is four species, in Ythan 96, as opposed to 124 species in the whole web). One can show that the ‘cost’ function *C_i* for the nodes of any three-level network is constrained to a narrow range, no matter what its internal structure, with exponents η that are compatible with the empirical range (Fig. 1a).

To prove this, we derive a general relationship between *C_i* and *A_i*. Let us start by considering a species *i* in the first level of a spanning tree of a three-level network. Species *i* sustains a sub-tree with *n₂*^{*i*} species in level 2 and *n₃*^{*i*} species in level 3, so that *A_i* = 1 + *n₂*^{*i*} + *n₃*^{*i*}, and *C_i* can be written as

$$C_i = A_i + \sum_{\text{level 2}} n_2^i A_j + \sum_{\text{level 3}} n_3^i A_j$$

All species in level 3 have *A_j* = 1, so the last sum yields *n₃*^{*i*}. The second sum provides the number of species sustained by all the *n₂*^{*i*} species (including them); that is, *n₂*^{*i*} + *n₃*^{*i*}. By introducing the average topological distance to species *i* of the species above *i*, namely *d_i* = (*n₂*^{*i*} + 2*n₃*^{*i*})/(*n₂*^{*i*} + *n₃*^{*i*}), then

$$C_i = A_i(d_i + 1) - d_i \tag{1}$$

This reasoning can be directly generalized to any network, so equation (1) is completely general.

For a three-level network, equation (1) provides narrow bounds for *C_i*. For a given value *A_i*, the smallest (or largest) *C_i* corresponds to the smallest (or largest) *d_i*. The shortest *d_i* occurs when all species above *i* are in the closest upper level, so that *d_i* = 1 and *C_i* = 2*A_i* − 1. The largest *d_i* corresponds to *i* in the first level, one species in level 2 and *A_i* − 2 species in level 3, yielding *C_i* = 3*A_i* − 3.

C_i is therefore bounded by two straight lines: 2*A_i* − 1 ≤ *C_i* ≤ 3*A_i* − 3.

Figure 1a shows that the 17 empirical food webs studied actually fall within this narrow region. The short range of empirical exponents (Table 1) is therefore simply a consequence of the small number of trophic levels in food webs. We have confirmed this by extensive analysis of three-level networks, for instance for random networks (Fig. 1b). Notice also that the concept of self-similarity is difficult to apply to networks with only three levels.

Garlaschelli *et al.* attempt to confirm their claim of universality by plotting *C₀* versus *A₀*. However, in our plot of *C₀* against *A₀* for the 17 food webs (Fig. 1c), we find a scaling with exponent 0.97 ± 0.10 (95% confidence), which

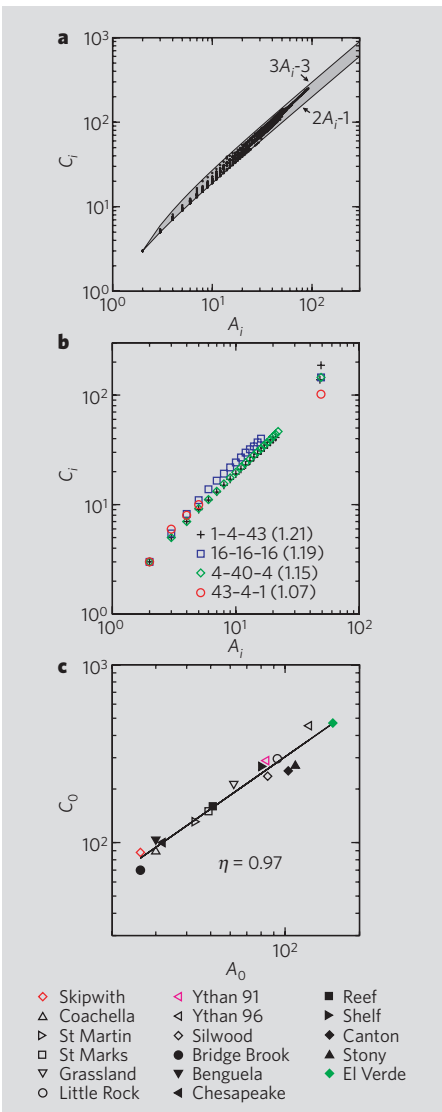


Figure 1 | Cost of energy transfer. The ‘cost’ function *C* plotted against *A*, the number of species that directly or indirectly feed on species *i*. **a**, *C_i* versus *A_i* plots for 17 empirical food webs (listed in Table 1), excluding the environment. All of them fall between the two limit cases (see text), showing that the exponent η is constrained to a small range close to unity for networks of any architecture, provided that they have only a few levels. **b**, Four random networks (designated by different symbols) with the same parameters as the St Marks food web (which has 3 trophic levels and 48 species) for various distributions of species within levels (as indicated by dashes in the key). Their exponents (in parentheses) are compatible with the empirical range. **c**, *C₀* versus *A₀* for the empirical food webs. The scaling is close to η = 1, as expected for networks with only a few trophic levels (see text). Plots in **a** and **b** were obtained after averaging *C_i* over 1,000 spanning trees chosen at random.

is significantly different from 1.13. We can show that an exponent very close to unity is expected for networks with only a few levels. In effect, equation (1) can be applied to the environment (node 0), yielding $C_0 = A_0(1 + d_0) - d_0$, where d_0 is the average distance of the species in the food web to the environment. Note that when d_0 is constant, one obtains a scaling $C_0 \propto A_0^\eta$, with $\eta = 1$. As empirical food webs mostly have three levels, the average distance has very little room to change, so it is expected to be roughly constant at $d_0 \approx 2$. The dispersion of data points around the straight line in Fig. 1c simply shows the variability of the average distances around $d_0 \approx 2$.

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FOOD-WEB TOPOLOGY

Garlaschelli et al. reply

Reply to: J. Camacho & A. Arenas *Nature* doi:10.1038/nature03839 (2005)

Although Camacho and Arenas¹ raise potentially interesting points, we believe that some of their arguments are flawed or undermined by poor statistics, and therefore that they do not invalidate our results².

Even though the two limiting curves shown in their Fig. 1a for three-level food webs define a 'narrow' region¹, several power laws can be drawn between them. The authors show for the randomized St Marks web (their Fig. 1b) that different distributions of species between levels yield different exponents, but they do not explain why the empirical web should display the particular value $\eta = 1.18$, which is only one of its allowed values. Moreover, as the (A_0, C_0) points in Fig. 1b are the most affected by the randomization, the allowed range for the C_0 versus A_0 curve in Fig. 1c must be even wider. In our opinion, the claim of Camacho and Arenas¹ that the observed values of η (including that for the C_0 versus A_0 curve) are due merely to the number of trophic levels is incorrect.

This means that our claim that allometric scaling adds information on food-web structure still stands, in particular with regard to the distribution of species between levels: for example, the distribution (6–31–11) for the real St Marks web is 'in between' two of

the randomized distributions (4–40–4 and 16–16–16) considered in Fig. 1b of Camacho and Arenas¹, and so the observed value ($\eta = 1.18$) lies between those for the two randomizations ($\eta = 1.15$, $\eta = 1.19$), but far from the other values. Randomized webs must therefore be forced to have a distribution of species between levels very similar to the empirical one in order to display (approximately) the same exponent.

What is more interesting is the broader range of exponents measured by Camacho and Arenas, suggesting that our results might be subject to variation if different webs are considered. However, we believe that the statistics are not strong enough for new conclusions to be drawn. The discrepancy between our results for some webs highlights the extreme sensitivity of η to small variations in the data, such as the presence or absence of even a single link, which can significantly affect the trophic-level structure.

The reason for this sensitivity is the small size of food webs, which is known to obscure the assessment of various other properties, such as the clustering coefficient and the degree distribution³. In this situation, the large-scale behaviour is best captured by the C_0 versus A_0 curve (Fig. 1c in ref. 1). However,

equation (1) of Camacho and Arenas¹ shows that, for $i = 0$, the leading term is $C_0 \propto A_0 d_0$, implying that, for the sublinear trend ($\eta = 0.97$) to hold, d_0 should decrease with the number of species. This is an unrealistic situation, again due to the small size of the webs, confirming that the statistics still yield no reliable result.

In the absence of data for larger webs, we can address only the expected dependence of d_0 on A_0 (or, equivalently, on N). In real webs³, d_0 is always very similar to the average distance l_{av} , which was shown⁴ to scale as $l_{av} \propto \ln(N)$ in empirical and model webs (including many of those considered by Camacho and Arenas). Then their equation (1) indicates that $C_0 \propto A_0 \ln(A_0)$, a curve that could be used as an alternative fit to the plots shown by Camacho and Arenas and by us; this corresponds to a different 'universality class', defined by the formal limit of infinite dimension D (logarithmic corrections naturally arise in such a limit) and representing an even more efficient topology.

Alternatively, it is possible — given that chain-length minimization reflects minimization of energy dissipation² — that d_0 is also related to the length l_{opt} of the optimal minimum-dissipation chain⁵. Depending on the system details, l_{opt} scales as $\ln(N)$, as $N^{1/3}$, or as a more general power law⁵.

The claims of Camacho and Arenas are therefore entirely based on the assumption that d_0 remains fixed as N increases, which in our view is an unrealistic hypothesis that disregards the wide range of possibilities described here.

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Somatic mosaicism in neuronal precursor cells mediated by L1 retrotransposition

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Revealing the mechanisms for neuronal somatic diversification remains a central challenge for understanding individual differences in brain organization and function. Here we show that an engineered human LINE-1 (for long interspersed nuclear element-1; also known as L1) element can retrotranspose in neuronal precursors derived from rat hippocampus neural stem cells. The resulting retrotransposition events can alter the expression of neuronal genes, which, in turn, can influence neuronal cell fate *in vitro*. We further show that retrotransposition of a human L1 in transgenic mice results in neuronal somatic mosaicism. The molecular mechanism of action is probably mediated through Sox2, because a decrease in Sox2 expression during the early stages of neuronal differentiation is correlated with increases in both L1 transcription and retrotransposition. Our data therefore indicate that neuronal genomes might not be static, but some might be mosaic because of *de novo* L1 retrotransposition events.

Neural stem cells give rise to three main lineages: astrocytes, oligodendrocytes and neurons. Most of the cells from these lineages are generated during development, although many continue to be born throughout life in the adult mammalian brain^{1–3}. In addition to the lineage decisions made by each cell type, many different types of cell are generated within each lineage. For example, within the neuronal lineage there are Purkinje, granule, pyramidal and basket cells, among others. Diversity also exists between individual cells within a cell type. Although incompletely understood, neuronal diversity is assumed to be governed by a combination of genetic and environmental factors^{4,5}. By studying neurogenesis, one can examine early fate choices between lineages, type differences within a lineage, and diversity between individual neurons.

The glycosylated form of cystatin C (CCg) allows the propagation of multipotent neural progenitor cells (NPCs) from either single cells or polyclonal cell populations plated at low density in the presence of fibroblast growth factor-2 (FGF-2). By examining the transcription profiles generated from CCg-responsive NPCs, we discovered a class of overexpressed transcripts corresponding to L1s.

L1s are abundant retrotransposons that comprise about 20% of mammalian genomes^{6–8}. Most L1s are retrotransposition-defective because they are 5' truncated, contain internal rearrangements or harbour mutations within their open reading frames^{9,10}. However, the average human genome is estimated to contain 80–100 retrotransposition-competent L1s (RC-L1s), and about 10% of these elements are classified as highly active or 'hot'¹¹. By comparison, the mouse genome is estimated to contain at least 3,000 active L1s^{12,13}.

Here we show that an engineered human L1 harbouring a retrotransposition indicator cassette can retrotranspose in adult rat NPCs *in vitro*, and in the brains of transgenic mice *in vivo*. Retrotransposition events in rat NPCs can influence both neuronal gene expression and differentiation *in vitro*. Retrotransposition events were detected in both neurogenic and non-neurogenic areas of the adult mouse brain, indicating that retrotransposition is not peculiar to mature NPCs but also occurs during neuronal development. Expression

analyses indicate that Sox2 might act to repress L1 transcription in rat adult hippocampus neural stem (HCN) cells. However, during neuronal differentiation, a decrease in Sox2 expression is correlated with a derepression of L1 transcription and an increase in L1 retrotransposition. These findings indicate that some neuronal genomes might not be static and might be influenced by *de novo* L1 retrotransposition events.

L1 transcripts are upregulated in CCg-responsive cells

CCg-responsive cells were isolated from heterogeneous HCN cells by plating them at low density in conditioned medium containing CCg (see Methods and Supplementary Methods for details). The resultant cell lines were then subjected to a microarray analysis to identify genes whose expression changed in response to culturing in CCg (Supplementary Fig. S1a and Supplementary Table S1). Surprisingly, a 1.5–2-fold enrichment of L1 transcripts in CCg-responsive cells was observed (Supplementary Fig. S1b and Supplementary Table S2). Semiquantitative reverse transcriptase polymerase chain reaction (RT-PCR) verified that L1 transcripts are enriched in CCg-responsive cells (Supplementary Fig. S1c).

Sox2 expression is inversely correlated with L1 promoter

We tested whether the promoter (that is, the 5' untranslated region (UTR)) of a retrotransposition-competent human L1 could function in rat NPCs. The human L1 5' UTR contains a YY1-binding site that is required for proper transcriptional initiation^{14,15} as well as two Sox-binding sites¹⁶ and a runt-domain transcription factor 3 (RUNX3)-binding site¹⁷. Sox proteins are expressed in a variety of tissues, including brain and testis¹⁸, and Sox2 is expressed in both embryonic stem cells and neural progenitor populations¹⁹. We therefore tested the effects of Sox2 on the human L1 5' UTR in HCN cells. Although Sox2 did not induce the expression of the reporter gene driven by the wild-type L1 5' UTR, Sox2 overexpression stimulated the expression of the reporter gene driven by a promoter that lacks the first 100 base pairs (bp) of the L1 5' UTR. The same trend was observed when the L1 promoter was cloned in an

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antisense orientation with reference to the gene encoding luciferase (Supplementary Fig. S2a). Taken together, these findings indicate that factors that interact with the first 100 bp of the human L1 5' UTR might be influenced by the Sox2 protein.

We next investigated the dynamics of L1 expression in HCN cells during neuronal differentiation. Sox2 expression was decreased during the first 24 h after the induction of differentiation and remained low for the duration of the 4-day assay (Fig. 1a). In contrast, L1 expression was stimulated up to tenfold during the

first 24 h after the induction of differentiation. It then decreased steadily from day 2 to day 4, but its expression remained higher than that observed in undifferentiated cells (day 0; Fig. 1b). As expected, the neuron-specific synapsin promoter²⁰ was induced gradually during neuronal differentiation (Supplementary Fig. S2b). Additional experiments showed that a short interfering RNA (siRNA)-mediated 75% decrease in Sox2 protein in HCN cells led to a sixfold increase in L1 expression (Fig. 1c), whereas mutation of the Sox-binding sites in the L1 5' UTR abolished the observed transcriptional repression by Sox2 protein (Fig. 1d). Last, chromatin immunoprecipitation (ChIP) revealed that Sox2 is associated with the endogenous rat L1 promoter in undifferentiated HCN cells (cultured with FGF-2) but is no longer associated with the rat L1 promoter 4 days after the induction of neuronal differentiation by FSK/RA (Fig. 1e). Consistently, histone deacetylase 1 (HDAC1) and methylation of histone H3 at Lys 9 (K9) (both associated with transcriptional silencing)²¹ were also present in undifferentiated HCN cells, whereas acetylation of H3 at K9 and its methylation at Lys 4 (K4) (associated with transcriptional activation) were present only in differentiated cells (Fig. 1e). Together, these data indicate that Sox2 represses L1 transcription in HCN cells and that a decrease in Sox2 expression during neuronal differentiation is correlated with chromatin remodelling, allowing a transient stimulation of L1 transcription.

Rat NPCs can support human L1 retrotransposition

HCN cells are a heterogeneous population composed of Sox2-positive neural stem cells (NSCs) and Sox2-negative NPCs. To test whether L1 can retrotranspose in HCN cells, we obtained L1 expression constructs harbouring a retrotransposition indicator cassette in their respective 3' UTRs. The indicator cassette consists of the gene encoding enhanced green fluorescence protein (EGFP) in the opposite orientation to the L1 transcript, a heterologous promoter (pCMV) and a polyadenylation signal (pA) (Fig. 2a). The EGFP gene is interrupted by an intron (IVS2 of the γ -globin gene) in the same transcriptional orientation as the L1 transcript. This arrangement ensures that EGFP-positive cells arise only when a transcript initiated from the promoter driving L1 expression is spliced, reverse-transcribed and integrated into chromosomal DNA, thereby allowing expression of the retrotransposed EGFP gene from the pCMV promoter^{22,23}.

Primary cultures of fresh rat adult hippocampus-derived neural progenitor (AHNP) cells, previously established HCN cells, rat primary neurons and astrocytes derived from the hippocampus, rat mesenchymal stem cells, rat fibroblasts, human CD34⁺ lymphocytes and human HeLa cells were electroporated with either an active L1 element (LRE3-EGFP) or a retrotransposition-defective L1 (JM111-EGFP) that contained two missense mutations in open reading frame 1 (ORF1)^{22–24}. Cells harbouring the L1 expression constructs were selected by the addition of puromycin to the culture medium, and puromycin-resistant cells were screened for EGFP expression by flow cytometry. After 3 days, EGFP expression was detected only in HeLa cells ($1.50 \pm 0.25\%$; data not shown). After 7 days, EGFP expression was detected in HCN cells ($0.75 \pm 0.07\%$) and AHNP cells ($1.50 \pm 0.50\%$). EGFP-positive cells were not detected in any of the other cell lines, even after several passages (Supplementary Fig. S3). PCR confirmed the presence of the retrotransposed (that is, spliced) EGFP gene in HCN-positive cells; sequencing of the PCR products confirmed the precise splicing of the intron (Fig. 2b; data not shown). Thus we conclude that a subset of cells present in the HCN and AHNP populations can support L1 retrotransposition.

To discriminate which kind of NPCs in the AHNP and HCN cell populations (that is, neuronal or glial progenitors) can support retrotransposition, individual EGFP-positive and EGFP-negative puromycin-resistant cells were collected by fluorescence-activated cell sorting (FACS) 7 days after electroporation. EGFP-positive cells

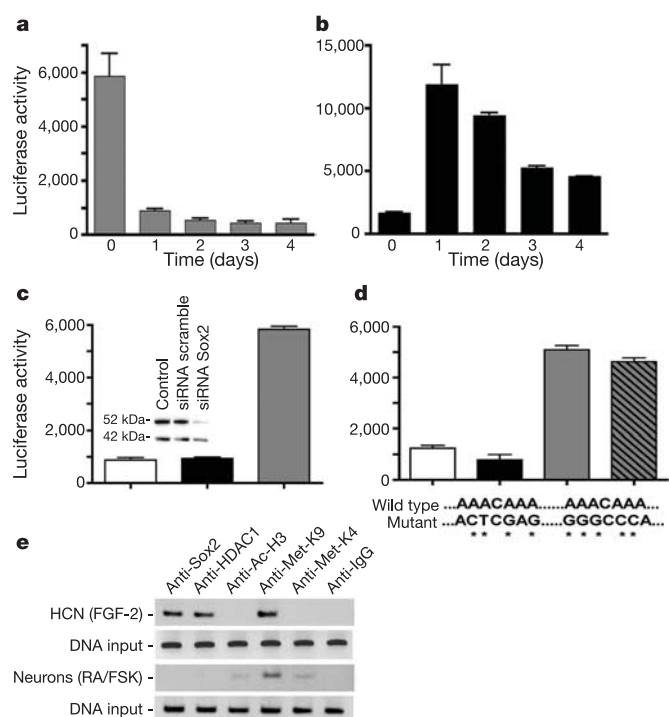


Figure 1 | L1 expression correlates with decreased Sox2 expression in HCN cells. **a**, Sox2 promoter is downregulated during neuronal differentiation. HCN cells were transfected with a luciferase reporter gene driven by the Sox2 promoter (day 0). The cells were then grown in N2 medium in the absence of FGF-2 but with RA/FSK to stimulate neuronal differentiation (days 1–4). **b**, HCN cells were treated as described in **a** and the expression of a luciferase reporter gene driven by the L1 5' UTR was followed for 4 days. **c**, siRNA inactivation of Sox2 transcripts correlates with increased activity in the L1 5' UTR promoter region. HCN cells were transfected with a luciferase reporter gene driven by a wild-type L1 promoter (control; white bar) in the presence of irrelevant siRNAs (siRNA scramble; black bar) or siRNA against the Sox2 mRNA (grey bar). Transfection of HCN cells with siRNAs against Sox2 mRNA decreased Sox2 protein levels by about 75% (inset). **d**, Mutations in the binding sites of Sox2 protein in the L1 5' UTR promoter region (grey bar) increased the luciferase activity in comparison with the intact L1 5' UTR (white bar) in HCN cells. Sox2 overexpression cannot induce the wild-type L1 5' UTR luciferase activity (black bar) but can activate the mutant L1 5' UTR (hatched bar). **e**, Recruitment of Sox2, HDAC1 and histone H3 modification on the endogenous rat L1 promoter region during neuronal differentiation by ChIP. Extracts of formaldehyde-fixed HCN cells were precipitated with specific antibodies, either in undifferentiated cells (FGF-2) or after induction to neuronal differentiation (RA/FSK), and then analysed by PCR with primers for the L1 5' UTR. Sox2 and HDAC1 were associated with the rat L1 promoter in undifferentiated cells only. Modifications of histone H3 associated with the L1 promoter indicate a dynamic chromatin structure, from a transcriptional silencing status (undifferentiated cells) to a transcriptional activation (neurons). Antibodies specific for methylated K9 in H3 (Met-K9), but not antibodies specific for methylated K4 (Met-K4) or acetylated K9 (Ac-H3), highly precipitated the L1 5' UTR sequence from HCN cell extracts. In contrast, antibodies specific for either Met-K4 or Ac-H3 precipitated the L1 5' UTR from neuronal extracts only. Error bars show s.e.m.

remained green during their first week in culture and often displayed a differentiated morphology when compared with cells in the initial HCN population (Fig. 2c, insets). However, EGFP expression was reduced over time and only a few cells remained EGFP-positive after 2 weeks in culture (Fig. 2c). Unexpectedly, all puromycin-resistant EGFP-negative clones also yielded a PCR product that corresponded in size to the retrotransposed EGFP gene (Fig. 2d). This observation might represent an event after FACS; a truncation of the 5' end or the retrotransposed EGFP gene might undergo epigenetic silencing either during or soon after L1 retrotransposition (see below).

Expression of EGFP in neuronal differentiation

To test whether clones harbouring L1 retrotransposition events remained multipotent, we stimulated their differentiation into the three main neural cell types: neurons, astrocytes and oligodendrocytes (see Methods for differentiation conditions). Despite containing a retrotransposed EGFP gene, all of the clones tested at the onset of this experiment were EGFP-negative, either because they never

expressed the retrotransposed EGFP gene ('negative clones') or because the retrotransposed EGFP gene was silenced during culturing in the presence of FGF-2 ('positive clones'). Under strong induction conditions, each clone tested was capable of undergoing differentiation into the three neural cell types at a similar rate to that of HCN cells (Supplementary Fig. S4a). Reactivation of the retrotransposed EGFP gene could be detected only during neuronal differentiation (Supplementary Fig. S4b). Time-lapse imaging revealed that EGFP expression began as early as 2 h after the induction of neuronal differentiation, indicating that it might have resulted from alterations in chromatin structure rather than new L1 retrotransposition events (Supplementary movie). EGFP-positive cells were rarely detected after the induction of astrocyte or oligodendrocyte differentiation, and they never colocalized with astrocyte (Gfap) or oligodendrocyte (Rip) markers, unlike the cells expressing the control CMV-driven EGFP cassette (Supplementary Fig. S4c), indicating that expression of the retrotransposed EGFP gene during neuronal differentiation in the analysed clones might be due to epigenetic modifications rather than to

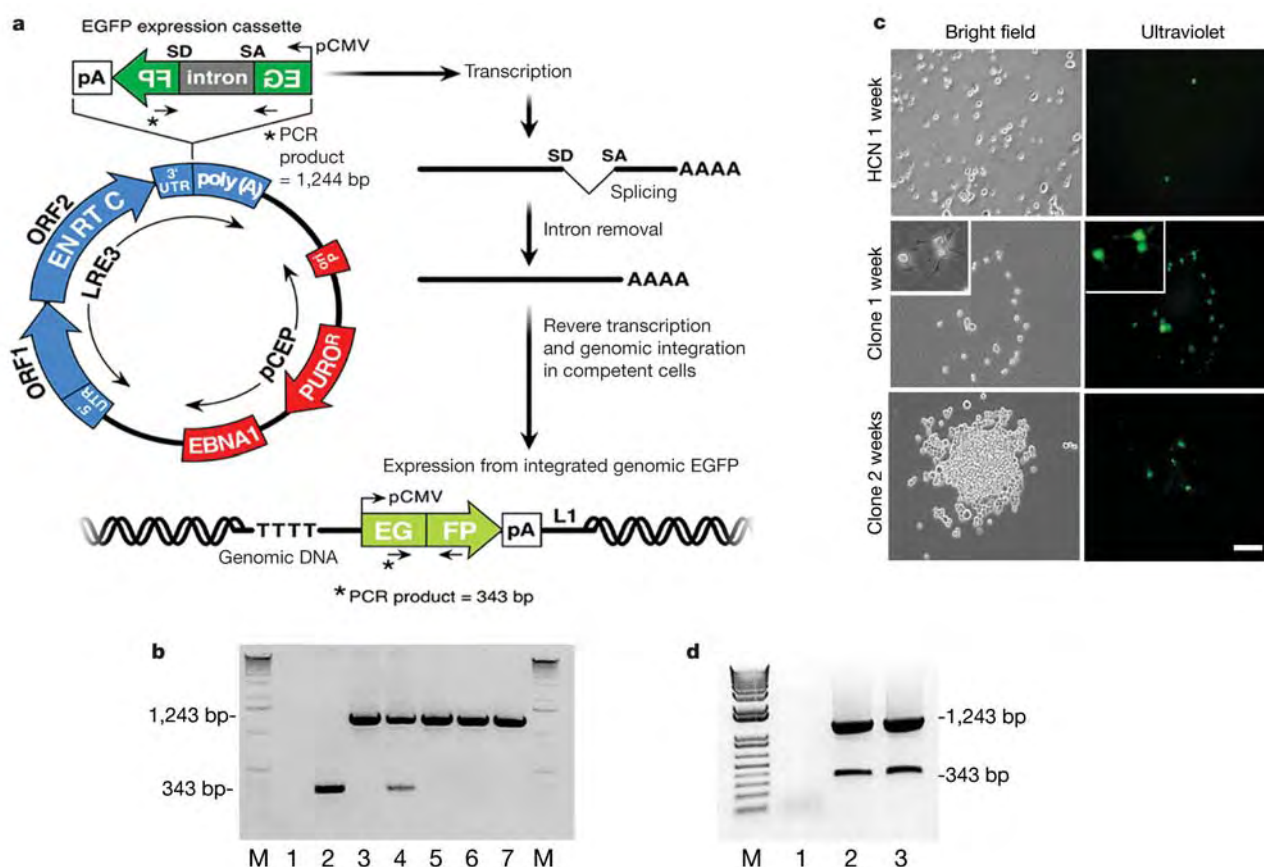


Figure 2 | NPCs can support L1 retrotransposition. **a**, Diagram of the L1-EGFP retrotransposition indicator construct. Retrotransposition-competent human L1s contain a 5' UTR harbouring an internal promoter^{14,15}, two open reading frames (ORF1 and ORF2; blue arrows), and a 3' UTR that ends in a poly(A) tail^{6,9}. The EGFP retrotransposition indicator cassette consists of a backward copy of an EGFP gene whose expression is controlled by the CMV minimal promoter (pCMV) and the thymidine kinase polyadenylation sequence (pA). The EGFP gene is also interrupted by an intron that is in the same transcriptional orientation as the L1. This arrangement ensures that EGFP expression will become activated only on L1 retrotransposition (see the text for details)^{22,23}. PCR primers flanking the intron in the EGFP gene are indicated at the bottom. The splice donor (SD) and splice acceptor (SA) sites of the intron are indicated. **b**, PCR analysis of genomic DNA isolated from different cell populations transfected

with LRE3-EGFP. PCR was conducted with the primers shown in **a**. The 1,243-bp PCR product corresponds to the original L1 vector harbouring the intron-containing EGFP indicator cassette. The 343-bp PCR product, diagnostic for the loss of the intron, indicates a retrotransposition event (lane 4). Lane M, molecular mass standards; lane 1, water; lane 2, positive control for the EGFP gene lacking an intron; lane 3, positive control for the EGFP gene containing the intron; lane 4, NPCs, 7 days after transfection; lane 5, NPCs, 3 days after transfection; lane 6, mesenchymal stem cells, 7 days after transfection; lane 7, fibroblast cells, 7 days after transfection. **c**, Despite containing a retrotransposed EGFP gene, cells that were initially EGFP-positive can no longer express EGFP. **d**, Puromycin-resistant clones that never expressed EGFP harbour L1 retrotransposition events, as indicated by the presence of the 343-bp PCR product. Lane M, molecular mass standards; lane 1, water; lane 2, EGFP⁺ clone; lane 3, EGFP⁻ clone.

neural cell-type-specific differences in pCMV promoter specificity.

To gain insights into the kinds of chromatin alterations that could be involved in EGFP gene reactivation during neuronal differentiation, we treated the clones with either 500 ng ml⁻¹ 5-azacytidine (5-Aza; a demethylating agent) for 4 days or 1 μ M trichostatin A (Tsa; an inhibitor of histone deacetylation activity) for 1 day in N2 medium. Both treatments caused an increase in EGFP expression in the different clones assayed. Moreover, immunostaining revealed that most of the EGFP-positive cells (63% in the Tsa treatment and 30% in the 5-Aza treatment) co-localized with Map2(a + b) but not with Gfap (Supplementary Fig. S4d–f), which is consistent with recent reports showing that epigenetic modifications accompany neuronal differentiation of NSCs^{21,25}.

L1s can insert into neuronally expressed genes

We next used inverse PCR²⁶ to characterize the post-integration sites from 17 clones (independent of the initial EGFP status to avoid expression bias) harbouring detectable L1 retrotransposition events by PCR. Interestingly, some of the insertions (clones B2, 22, 2, 10, 6 and 5; Table 1) occurred within neuronally expressed genes (see Supplementary Information). Comparison of the post-integration sequence with the pre-integration site present in the University of California Santa Cruz genome database revealed that the characterized retrotransposition events occurred by conventional

endonuclease-dependent retrotransposition²⁶ (Supplementary Information). Curiously, clones 2 and 5 contained independent L1 retrotransposition events into the *Slc6a6* gene (Table 1). Although the number of characterized insertions is relatively small, our data indicate that L1 retrotransposition events can occur in neuronally expressed genes. The ability of L1 to retrotranspose into genes is consistent with results from previous studies performed in transformed cultured cell lines^{27–29}.

Retrotransposition events in NPCs can affect cell fate

In a mixed differentiation protocol (Supplementary Fig. S4a), most HCN clones harbouring detectable L1 retrotransposition events had a tendency to differentiate into neurons rather than glial cells (Table 1). To test whether new L1 retrotransposition events could influence the phenotype of the NPCs *in vitro*, we fully characterized the insertion site present in clone 22 (Cl 22). The L1 was inserted, in the antisense orientation, into the 5' UTR of the rat neuronal *chapsyn110/Psd-93* gene (Fig. 3a). RT-PCR with primers specific for *Psd-93* revealed that RNA levels were about tenfold higher in Cl 22 cells than in the original HCN population. Moreover, *Psd-93* was not overexpressed in Cl 28, which contains two different L1 insertions (Fig. 3b). The *Psd-93* protein level was also 3–5-fold higher in Cl 22, and the increase in *Psd-93* protein level was correlated with a decrease in Sox2 expression (Fig. 3c).

Table 1 | Analyses of L1 insertions in HCN cell-derived clones

Clone	Initial EGFP expression	Phenotype	Locus	L1 insertional target site description
HCN	Negative		-	-
B2	Negative		1q43	Inside olfactory receptor <i>Olr346</i>
3	Positive		2q12	Inside <i>Dhfr-1</i> (dehydrofolate reductase-1)
28	Positive		6q24 Xq22	Inside pseudogene similar to human glyceraldehydes-3-phosphate dehydrogenase (<i>G3PDH</i>) Inside <i>Loc317538</i> , similar to dystrophin major muscle isoform
B9	Positive		3q34	150 kb distant from gene similar to brain type ryanodine receptor 3 (<i>RyR3</i>), a Ca ²⁺ release channel, and 55 kb distant from gene similar to the Notchless WD repeat
22	Positive		1q32	Inside <i>Chapsyn110</i> , postsynaptic protein 93 (<i>Psd-93</i>)
7	Positive		18q12.3	25 kb distant from similar to human <i>G3PDH</i>
2	Positive		4q34	Inside <i>Slc6a6</i> (solute carrier family 6), neurotransmitter transporter, taurine
F10	Negative	n.d.	1q31	2 kb distant from a number of predicted genes with unknown function
9	Negative	n.d.	19q11	Inside <i>Loc364967</i> , predicted protein similar to spermatogenesis associated glutamate-rich protein 4f from <i>Mus musculus</i>
6	Negative		6q16	Inside <i>NVP-3</i> , neural visinin-like Ca ²⁺ -binding protein type 3
5	Positive		4q34	Inside <i>Slc6a6</i> (solute carrier family 6), neurotransmitter transporter, taurine
26	Positive		2q12	60 kb distant from gene similar to <i>Xrcc4</i> DSB repair
13	Positive		3p13	20 kb distant from <i>Nidogen 2</i> , a base membrane protein involved in cell adhesion
21	Positive		2q32	50 kb distant from hypothetical kazal type serine protease inhibitor domain, with EF-hand Ca ²⁺ binding and N-CAM L1 motif
B5	Negative	n.d.	19q11	Inside hypothetical protein FLJ10846-like, with unknown function, and 30 kb distant from CG9882-PA with TPR and ribosomal protein L11 methyltransferase domains
10	Positive		18p11	Inside <i>rCNR</i> gene, a cadherin-related neuronal receptor
1	Positive		7q33	Inside <i>LRTP</i> , similar to mouse testis specific protein with leucine-rich repeat domain and involved in regulation of protein phosphatases

Phenotypes were determined by exposing the clones to mixed differentiation (RA/FBS) medium, and L1 target sites were determined by inverse PCR (see Methods). Percentages of oligodendrocytes in these clones were too low to display or nonexistent (data not shown). White, undifferentiated; black, neurons (Map2(a + b)); grey, astrocytes (Gfap). n.d., not determined.

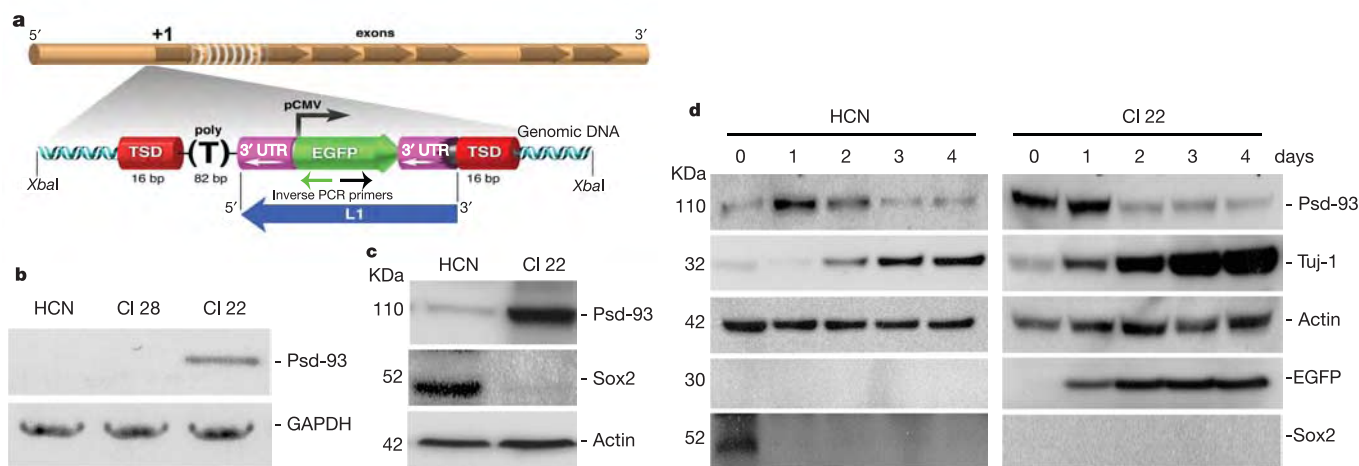


Figure 3 | L1 retrotransposition events can modify neuronal gene expression. **a**, Schematic representation of an L1 insertion into the *Chapsyn110/Psd-93* gene. The primers used in the inverse PCR experiments are indicated by the green and black arrows. **b**, RT-PCR showed that *Psd-93* expression is higher in the clone harbouring the L1 insertion in the *Psd-93* gene (CI 22) than in naive HCN cells, or another cell line harbouring L1 insertions at different loci (CI 28). GAPDH, glyceraldehyde-3-phosphate dehydrogenase. **c**, Western blot analysis revealed a higher expression of *Psd-93* protein but a lower expression of Sox2 protein in CI 22

cells than in naive HCN cells. **d**, Kinetic analysis of *Psd-93* expression during neuronal differentiation. In HCN cells, *Psd-93* was upregulated during early stages of neuronal differentiation (day 1) and then decreased over the course of the 4-day assay. By comparison, in CI 22, *Psd-93* was expressed initially at high levels (day 0) and was upregulated to a smaller extent on neuronal differentiation (day 1). However, the decrease in expression mirrored that seen in naive HCN cells (days 2–4). As controls, we monitored the expression of actin, EGFP, Tuj-1 and Sox2 protein levels in both cell lines.

We next examined the expression of *Psd-93* during neuronal differentiation. In HCN cells, *Psd-93* protein was induced during the early stages (24 h) of neuronal differentiation, at a similar time as the retrotransposed EGFP gene was being reactivated and Sox2 was downregulated. *Psd-93* protein levels then decreased during neuronal maturation until they reached a baseline expression level on day 4 of the assay (Fig. 3d). By comparison, in CI 22 cells, the initial level of *Psd-93* protein was higher than that observed in HCN

cells and, after differentiation, was induced to a smaller extent than in HCN cells. However, the downregulation of the *Psd-93* protein after 48 h was similar to that observed in HCN cells (Fig. 3d). Thus, despite its initial overexpression, further regulation of the *Psd-93* gene activity in CI 22 remained unaffected, indicating that the entire locus might have been subject to higher-order regulation.

To verify whether the initial overexpression of *Psd-93* in CI 22 affected neuronal differentiation, we used specific siRNAs to

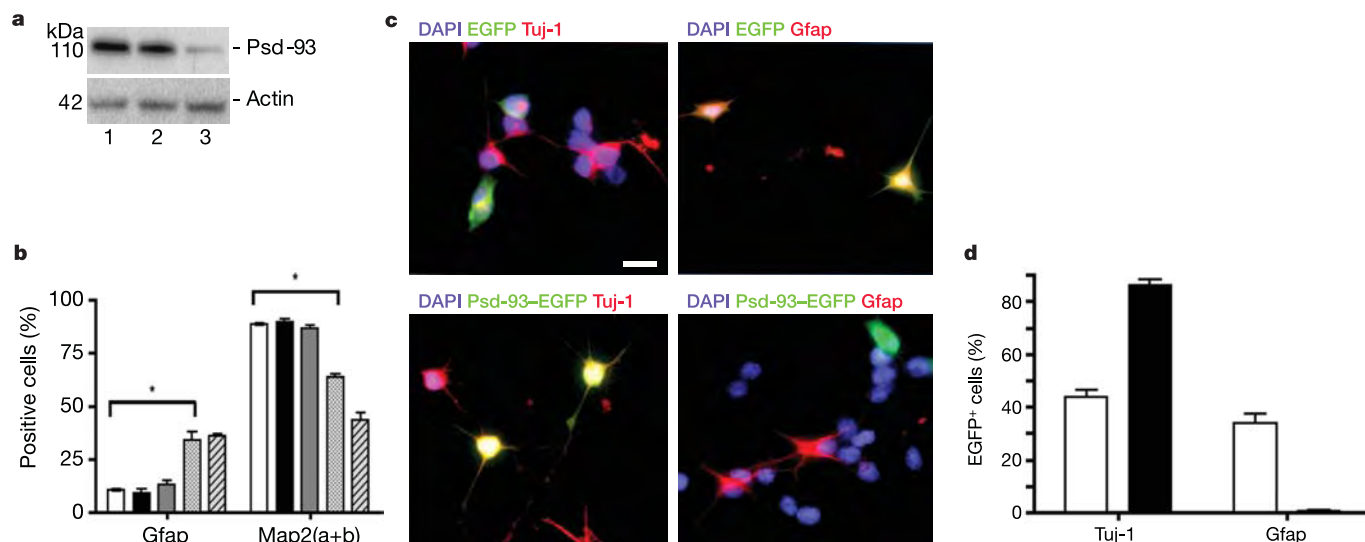


Figure 4 | An L1 retrotransposition event can drive neuronal maturation through *Psd-93* overexpression. **a**, siRNA against *Psd-93* transcripts (lane 3) results in a 70% decrease in *Psd-93* protein levels in CI 22 cells (lane 2, without siRNA). Control cells (lane 1) were treated with non-specific siRNAs. **b**, Both CI 22 cells (white bars) and CI 22 cells treated with irrelevant siRNAs (black and grey bars) have a tendency to differentiate into neurons when plated in mixed (RA/FBS) differentiation medium. By comparison, lower levels of *Psd-93* transcripts (stippled bars) attenuated the strong neuronal bias of CI 22 cells to that observed in naive HCN cells (hatched bars) (* $P = 0.0056$). **c**, *Psd-93* overexpression induced neuronal

differentiation/maturation of HCN cells plated in RA/FBS. The *Psd-93*-EGFP fusion protein co-localizes with a neuronal marker (Tuj-1) but not with an astrocyte marker (Gfap; shown in red). EGFP expression is shown in green. Co-localization of *Psd-93*-EGFP and Tuj-1 is shown in the lower left panel. DAPI, 4,6-diamidino-2-phenylindole. Scale bar, 10 μ m. **d**, Quantification of panel **c**. EGFP-positive (fused (black bars) or not (white bars) with *Psd-93*) cells were scored for their co-localization with Tuj-1 or Gfap markers. The percentage of each cell type is indicated on the y axis. Error bars in all panels show s.e.m.

transiently downregulate *Psd-93* mRNA transcripts (Fig. 4a). Remarkably, 4 days after electroporation, the resultant Cl22 cells seemed less differentiated and showed a decrease in cellular processes (data not shown), and protein levels more closely resembled those of naive HCN cells (Fig. 4b). Consistent with this notion was our observation that overexpression of a *Psd-93-egfp* fusion protein in HCN cells was able to induce neuronal differentiation under the mixed condition RA/FBS (Fig. 4c, d). Together, these observations indicate that retrotransposition of an engineered human L1 into this gene can cause an alteration in gene expression, which results in a phenotypic change in cell behaviour.

L1 somatic retrotransposition in mouse brain

To determine whether L1 retrotransposition can occur *in vivo*, we generated a transgenic animal harbouring a retrotransposition-competent human L1 element ($L1_{RP}$) under the control of its endogenous promoter^{23,30}. The EGFP reporter gene is under control of the CMV promoter; thus, the retrotransposed EGFP gene has the potential to be expressed ubiquitously in mice. We obtained two independent founders (Fo4 and Fo6) containing the $L1_{RP}$ /EGFP transgene. They were bred with wild-type C57BL/6J mice, and the resultant progeny were screened for L1 retrotransposition events by genotyping tail DNA with PCR primers flanking the intron present in the EGFP retrotransposition indicator cassette²³. Both founders were positive for the transgene. Figure 5a is a diagram of the progeny of Fo4. Animal 1 lacked both the $L1_{RP}$ /EGFP transgene and a retrotransposed EGFP gene. Animals 2 and 3 contained only the

$L1_{RP}$ /EGFP transgene. Animal 4 lacked the $L1_{RP}$ /EGFP transgene but contained a retrotransposed EGFP gene. Animal 5 contained both the $L1_{RP}$ /EGFP transgene and the retrotransposed EGFP gene.

The progeny (a total of seven) containing only the $L1_{RP}$ /EGFP transgene from both founders (for example animals 2 and 3; Fig. 5a) were selected for further analysis. We killed adult animals and used anti-EGFP antibodies to detect L1 retrotransposition events by immunofluorescence microscopy in tissues from different organs. EGFP-positive cells were detected in germ cells (ovary and testes, previously shown to express L1 ORF1³¹) but not in other somatic tissues (Supplementary Figs S5 and S6). EGFP-positive cells also were found in the brains of both male and female transgenic animals (for example striatum, cortex, hypothalamus, hilus, cerebellum, ventricles, amygdala and hippocampus; Fig. 5c–k). EGFP-positive cells co-localized only with a neuronal marker (NeuN) and not with oligodendrocyte (glutathione S-transferase π , GST π) or astrocyte (S100- β) markers, indicating that L1 retrotransposition might have occurred in neuronal precursor cells rather than glial precursor cells or a common precursor cell early during embryogenesis (Fig. 5i–k). Because the migration and maturation of the different brain EGFP-positive cell types occurred in distinct regions in the wild-type brain, L1 retrotransposition most probably occurred in several distinct NPCs at different times during brain development.

Analysis of embryos at embryonic day 8.5 (E8.5) showed no EGFP-positive cells. At this stage the neural tube is already defined and separated from tissues with the same embryonic origin (such as the skin). By comparison, EGFP-positive cells were detected in the

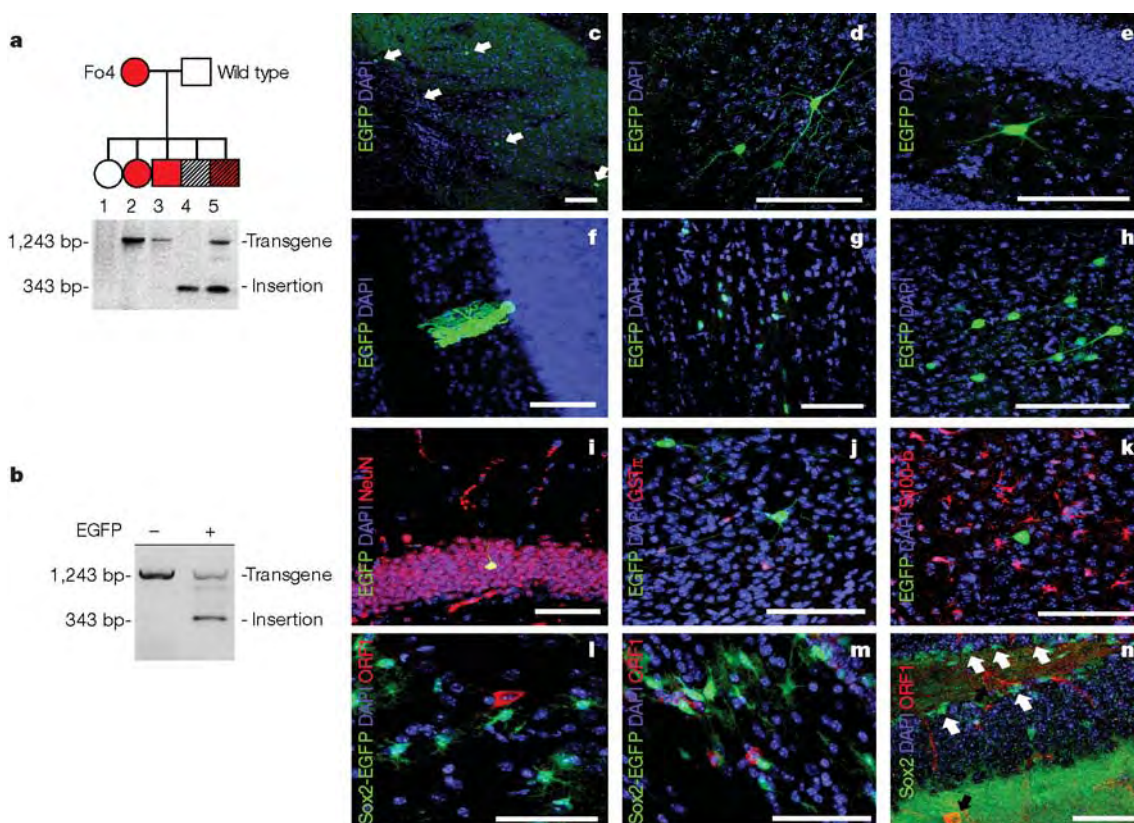


Figure 5 | L1 retrotransposition detection in the brains of transgenic mice. **a**, Genotyping results on tail DNA from offspring of founder Fo4 (animals 1–5). The PCR primers detected the two possible EGFP configurations²³. Red circles (female) and red squares (male) indicate animals containing the $L1_{RP}$ /EGFP transgene; shading indicates animals containing the retrotransposed EGFP gene. **b**, PCR on genomic DNAs isolated from laser-captured cells. About 50 cells were collected in each population (EGFP-positive and EGFP-negative) from the brains of two

animals harbouring the $L1_{RP}$ /EGFP transgene. EGFP-positive cells were detected in different regions of the mouse brain, such as striatum (**c**, **l**) (white arrows in **c** indicate EGFP-positive cells), cortex (**d**), hypothalamus (**k**), hilus (**e**), cerebellum (**f**), ventricles (**g**, **l**, **m**), amygdala (**h**) and hippocampus (**i**, **n**). ORF1-positive cells were also found in different regions of the brain, such as the ventricular zone (**l**, **m**) and the dentate gyrus of the hippocampus (**n**; white arrows indicate Sox2-positive cells, black arrows indicate ORF1-positive cells). Scale bar, 10 μ m.

cephalic neural tube but not in other regions or organs of the E10.5 embryo. Taken together, these results indicate that L1 retrotransposition might take place during both embryonic and adult neurogenesis (Supplementary Fig. S5).

To confirm that the brain cells contained L1 retrotransposition events, we next isolated EGFP-positive and EGFP-negative cells by laser-capture microscopy and subjected genomic DNAs extracted from those cells to PCR analysis (Fig. 5b). EGFP-positive cells contained both the L1_{RP}/EGFP transgene and the retrotransposed EGFP gene. Sequence analysis confirmed that the 343-bp PCR product is the precisely spliced EGFP gene (data not shown). EGFP-negative cells contained the L1_{RP}/EGFP transgene and a very faint PCR product corresponding to the spliced EGFP gene. This faint amplicon might represent silenced EGFP-gene insertions from mature neurons.

Finally, consistent with the hypothesis that Sox2 acts to repress L1 expression in NSCs and that a decrease in Sox2 expression in NPCs is correlated with an increase in L1 expression and retrotransposition, our initial experiments show that anti-Sox2 antibodies do not co-localize with cells that stain positively with a mouse anti-ORF1p antibody in Sox2-EGFP transgenic animals³² (Fig. 5l, m) or wild-type C57BL/6J brain sections (see Fig. 5n, for example).

Discussion

Previous studies have indicated that L1 retrotransposition can occur in germ cells or in early embryogenesis, before the germ line becomes a distinct lineage^{33,34}, whereas a cultured cell retrotransposition assay has revealed that human and mouse L1 elements can retrotranspose in a variety of transformed or immortalized cultured cell lines^{22,26,35}. There is only one documented example of a genuine somatic retrotransposition event *in vivo*, and it occurred into the adenomatous polyposis coli gene of a colorectal tumour³⁶. Our data show that rat NPCs can support retrotransposition of an engineered human L1 and that a human L1 element can undergo somatic retrotransposition in the mouse brain. We were unable to detect L1 insertions in other somatic tissues, indicating that the frequency of L1 retrotransposition might be lower in those tissues than in NPCs.

Two previous reports documented the retrotransposition of an engineered human L1/EGFP transgene in mice. Ostertag *et al.* demonstrated that a human L1 element could retrotranspose in the male germline before the onset of meiosis II³³. However, because the sperm-specific acrosin promoter drove the expression of the retrotransposed EGFP gene, it is unlikely that these authors would have been able to detect the expression of the EGFP protein in the brain. Prak *et al.*³⁴ used the same L1_{RP}/EGFP transgene described in this study, and the authors detected EGFP-positive cells in the testes but not in the brain or other tissues. However, despite detecting a low level of EGFP mRNA expression in a variety of different tissues by RT-PCR³⁴, the authors only analysed EGFP expression directly; they did not use anti-EGFP antibodies to detect protein expression.

Our expression analyses lead us to propose that L1 retrotranspositions are silenced in NSCs growing in FGF-2 owing to Sox2 repression. Downregulation of Sox2 triggered by CCG might lead to chromatin modifications. Indeed, our inability to find overexpressed neuronal genes in NPCs, coupled with the finding that all the clones selected for retrotransposition remain multipotent, indicates that L1 can retrotranspose during early stages of neuronal differentiation.

Our results are consistent with previous analyses that retrotransposition events generated from an engineered human L1 often insert into genes^{27–29}. Indeed, we found that some L1 retrotransposition events in NPCs integrated into genes that are expressed in neurons, whereas other insertions were located in gene 'deserts' and/or repetitive sequences. In one instance, we have shown that retrotransposition of an engineered human L1 into the *Psd-93* gene can lead to its overexpression, which influences the differentiation pattern of the NPCs. These data provide proof in principle that new L1 retrotransposition events can affect the expression of neuronal

genes *in vitro*. As indicated from previous analyses^{35,37}, we predict that L1 insertions into genes may also repress their expression or may lead to alternative splicing patterns³⁸.

Thus, our findings indicate that an engineered human L1 can retrotranspose in rat NPCs and indicate that individual neurons might be mosaic with respect to L1 content. Future experiments will focus on whether endogenous L1s naturally retrotranspose in NPCs and whether this process has any developmental significance. However, with those caveats being clearly stated, it is tempting to speculate that some of the genomic changes necessary for the uniqueness of individuals within a population, as defined by their neural circuitry, might be driven, in part, by the activities of mobile elements.

METHODS

Cell culture and transfection. HCN-A94 and freshly isolated AHNPCs cells were prepared and cultured as described³⁹. Plasmid transfections were performed with Fugene6 (Roche) for luciferase assay and with a rat NSC nucleofector electroporation kit in all other experiments in accordance with the manufacturer's instructions (Amaxa Biosystem).

Luciferase assay and siRNA sequences. Luciferase activity was measured with the Dual-Luciferase reporter assay system (Promega) in accordance with the manufacturer's protocol. The L1 5' UTR-Sox2 mutant plasmid was a gift from J. Athanikar. The Sox2 promoter and complementary DNA were gifts from A. Rizzino. The Synapsin-1 promoter region was a gift from G. Thiel. The *Psd-93-egfp* plasmid was a gift from D. Bredt.

ChIP. The ChIP assay was performed essentially in accordance with the manufacturer's protocol with a ChIP assay kit (Upstate) and primers for the rat L1 5' UTR promoter region.

Immunofluorescence and immunoblotting. Immunofluorescence was performed as described previously⁴⁰. The mouse polyclonal ORF1 antibody was a gift from S. L. Martin. Western blotting was performed with standard protocols.

Expression profile analyses. To normalize for processing errors, each replicate consisted of cells that were plated at the same time from the same starting bulk population. The following cells were profiled: CCG-responsive cells, neurons and astrocytes (twenty 96-well plates). The resulting cRNA from each sample was hybridized to DNA microarrays (Affymetrix Rat Genome RG-U34A). Data analysis was performed with Affymetrix MAS4.0 software to search for transcripts differentially expressed in CCG-responsive NPCs in comparison with proliferating bulk progenitors and differentiated cells. The results were analysed with three different data mining tools: Bullfrog, an empirically based filtering algorithm⁴¹, dChip software for model-based expression analysis⁴², and Felix Naef's statistically based perfect-match-only algorithm⁴³.

RT-PCR analysis. Total RNA was prepared with an RNAeasy kit (Qiagen). First-strand cDNA synthesis was performed with the Superscript II kit (Invitrogen). PCR was performed with *Taq* polymerase (BMH).

Retrotransposition assays. Antibiotic selection (puromycin, 1 µg ml⁻¹) was begun 48 h after electroporation. After 7 days, transfected puromycin-resistant cells were analysed with a Becton Dickinson FACStar Plus containing a blue argon laser (488 nm) and fluorescein filter sets (530/30 nm bandpass). The EGFP PCR primers used here were as described previously²³. The L1 retrotransposition cassettes were gifts from H. H. Kazazian Jr.

Inverse PCR. Genomic DNAs were digested with *SspI* or *XbaI*, extracted with phenol and then chloroform, and subjected to overnight ligation. The products then were re-extracted, ethanol-precipitated and subjected to first-round PCR amplification with the primers for the EGFP expression cassette. The L1 pre-integration sequence was identified with Blast (<http://www.ncbi.nlm.nih.gov/BLAST/>) and the Celera database (<http://www.celera.com/celera-discovery/system.com/>).

L1 transgenic animals. Transgenic mice were generated with the standard pronuclear injection protocol⁴⁴. The potential founders were screened by PCR by using primers described previously²³. The L1_{RP}/EGFP construct was a gift from H. H. Kazazian Jr.

Tissue preparation. Animals were killed with an overdose of anaesthetics and perfused transcardially with 4% paraformaldehyde in phosphate buffer (0.1 M, pH 7.4). Brains were cut coronally (40 µm) on a sliding microtome from a solid-CO₂-cooled block before use.

Other methods. More details can be found in the Supplementary Methods.

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Author Information Microarray data have been deposited in the GEO archive under accession number GSE2499, and the CI22 L1 insertion sequence has been deposited in GenBank under accession number AY995186. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to F.H.G. (gage@salk.edu).

Nucleotide-dependent bending flexibility of tubulin regulates microtubule assembly

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The atomic structure of tubulin in a polymerized, straight protofilament is clearly distinct from that in a curved conformation bound to a cellular depolymerizer. The nucleotide contents are identical, and in both cases the conformation of the GTP-containing, intra-dimer interface is indistinguishable from the GDP-containing, inter-dimer contact. Here we present two structures corresponding to the start and end points in the microtubule polymerization and hydrolysis cycles that illustrate the consequences of nucleotide state on longitudinal and lateral assembly. In the absence of depolymerizers, GDP-bound tubulin shows distinctive intra-dimer and inter-dimer interactions and thus distinguishes the GTP and GDP interfaces. A cold-stable tubulin polymer with the non-hydrolysable GTP analogue GMPCPP, containing semi-conserved lateral interactions, supports a model in which the straightening of longitudinal interfaces happens sequentially, starting with a conformational change after GTP binding that straightens the dimer enough for the formation of lateral contacts into a non-tubular intermediate. Closure into a microtubule does not require GTP hydrolysis.

The dynamic behaviour of microtubules is crucial to their functions, and although regulated by cellular factors during the cell cycle, it is an intrinsic property of the tubulin subunit^{1–3}. The binding, hydrolysis and exchange of nucleotide have been identified as central to the conformational flexibility that tubulin exhibits in

its polymerization–depolymerization cycle. Structures of two different assembly states of tubulin are available at atomic resolution: tubulin in a polymerized, straight protofilament bound to the stabilizer Taxol (obtained by electron crystallography of zinc-induced sheets^{4,5}), which very closely corresponds to that of tubulin in

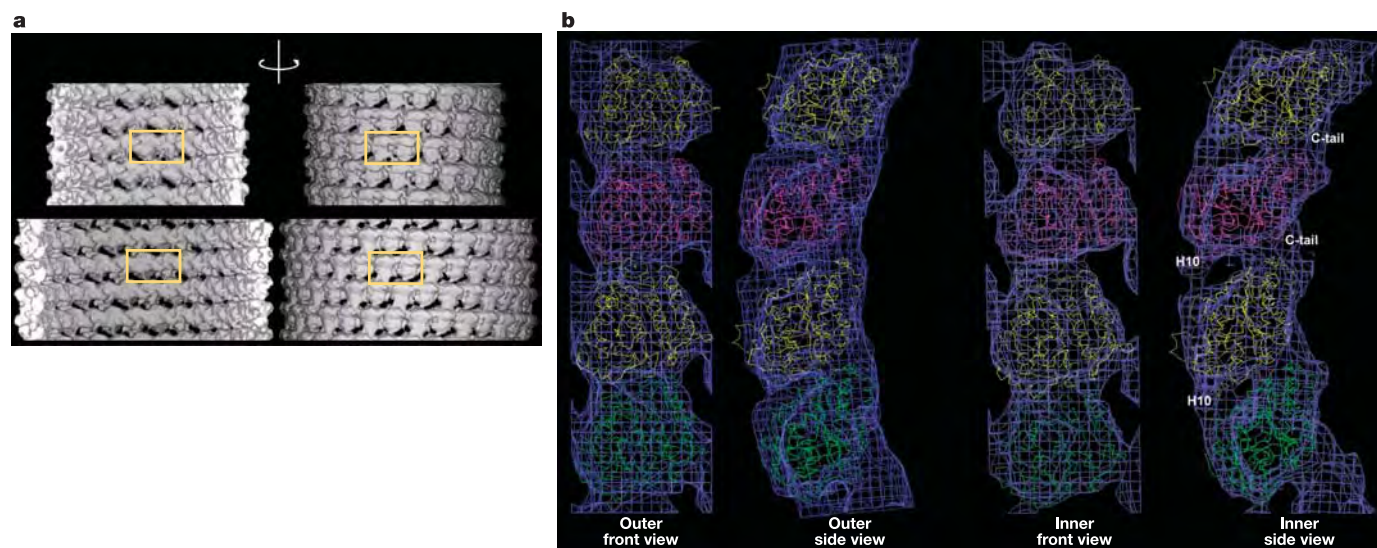


Figure 1 | Cryo-EM reconstruction of double-layered tubes of GDP-bound tubulin and docking of crystallographic models. **a**, Three-dimensional densities for the inner (top) and outer (bottom) layers of the GDP-tubulin tubes. The inside view of the tubes is shown on the left, the outside view on the right. Dimer boundaries are indicated by yellow boxes. **b**, The

crystallographic structures of tubulin were manually docked into the cryo-EM densities of the outer and inner layers of the tubes. β -Tubulins (1SA0) are shown in yellow, α -tubulins (1JFF) in green for the lower dimer and in magenta for the top dimer. Major regions of discrepancy with the crystal structures are indicated in the rightmost panel.

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microtubules⁶; and tubulin in a depolymerized, curved conformation bound to a fragment of the stathmin homologue RB3 and colchicine (obtained by X-ray crystallography)⁷. These distinctly different structures have the same nucleotide content, in both cases the conformation of GTP-bound α -tubulin and of GDP-bound β -tubulin are the same, and intra-dimer and inter-dimer contacts are practically indistinguishable. Here we present the structure of GDP-bound tubulin in the absence of depolymerizing agents and show distinctive intra-dimer and inter-dimer interactions. But the question still remained: how does the binding of GTP to the exchangeable site (E-site) affect the bending of the dimer, the dimer–dimer interface and the microtubule assembly? We have addressed this question by characterizing the assembly of tubulin bound to the non-hydrolysable GTP analogue GMPCPP into ribbon structures at low temperatures. We propose that these structures correspond closely to the open sheets revealed at the growing ends of microtubules⁸.

Structure of GDP-tubulin in the absence of depolymerizers

Divalent cations facilitate the self-assembly of GDP-tubulin into ring-like structures of curved protofilaments^{9,10} and stabilize the protofilament peels observed at depolymerizing microtubule ends^{11,12}. We found conditions under which closure does not happen and the protofilaments keep turning in a tight, one-start helix (Supplementary Information). The most common arrangement is a double-layered tube with 32 tubulin monomers in one turn of the outer layer and 24 in the inner layer (Supplementary Fig. S1a and Supplementary Table S1). Cryo-EM images show diffraction up to 17-Å resolution (Supplementary Figs S1b and S2). The double-layer character of the tubes results in systematic overlap of the Bessel terms (Supplementary Fig. S2) and makes it impossible to use traditional helical reconstruction¹³. We developed an iterative Fourier Bessel method by which the relative orientation of different tubes can be determined and used to produce a three-dimensional reconstruction¹³. We have implemented this method to obtain a reconstruction of GDP-tubulin at 12 Å resolution.

The averaged data set of 19 images shows significant signal up to 10 Å resolution axially (Supplementary Fig. S1c; some averaged layer lines in Supplementary Fig. S3). Layer lines corresponding to the $\alpha\beta$ dimer are noticeable, already indicating the presence of differences between monomers and dimers. The three-dimensional densities for the two layers were reconstructed independently using data up to 12 Å resolution (Fig. 1a). Fourier shell correlation showed that the dimer densities from both layers are the same up to 15 Å (not shown) and thus that the GDP-tubulin dimer has basically the same conformation in the two layers, their different curvature arising from different inter-dimer contacts.

In all the tubulin polymers characterized so far at medium to high resolution, zinc-sheets, microtubules and the RB3/colchicine–tubulin complex^{5–7}, α -tubulin and β -tubulin are very similar to each other and intra-dimer and inter-dimer interfaces, which have different nucleotide contents (the intra-dimer interface contained a non-exchangeable GTP, whereas the inter-dimer interface contained GDP at the E-site), are almost indistinguishable. It has been proposed that the conformation of GDP-bound tubulin in a microtubule lattice (or zinc-sheet) would be constrained so as to resemble that when bound to GTP (the free energy for hydrolysis being stored in the microtubule lattice as mechanical constraint)^{14–16}. In contrast, the RB3/colchicine-bound structure could be affected by the binding of these ligands. In our structure of unconstrained, GDP-bound tubulin the intra-dimer and inter-dimer interfaces are significantly different (Fig. 1a and Supplementary Figs S2 and S3). To compare the monomer structure and the subunit organization in our GDP-tubulin polymer with those found in previous studies we docked the atomic models of tubulin into our reconstructions (Fig. 1b). The overall fit of the monomer is good, but there is extra density at the carboxy terminus of both subunits in both layers that corresponds to the C-terminal amino acids disordered in the crystals but contributing significantly to our 12-Å reconstruction. In addition, a distinctive conformational change occurs, as the interfaces open but the interaction between helix H10 in one monomer and helix H6 in the other is maintained. Although the conformation of our

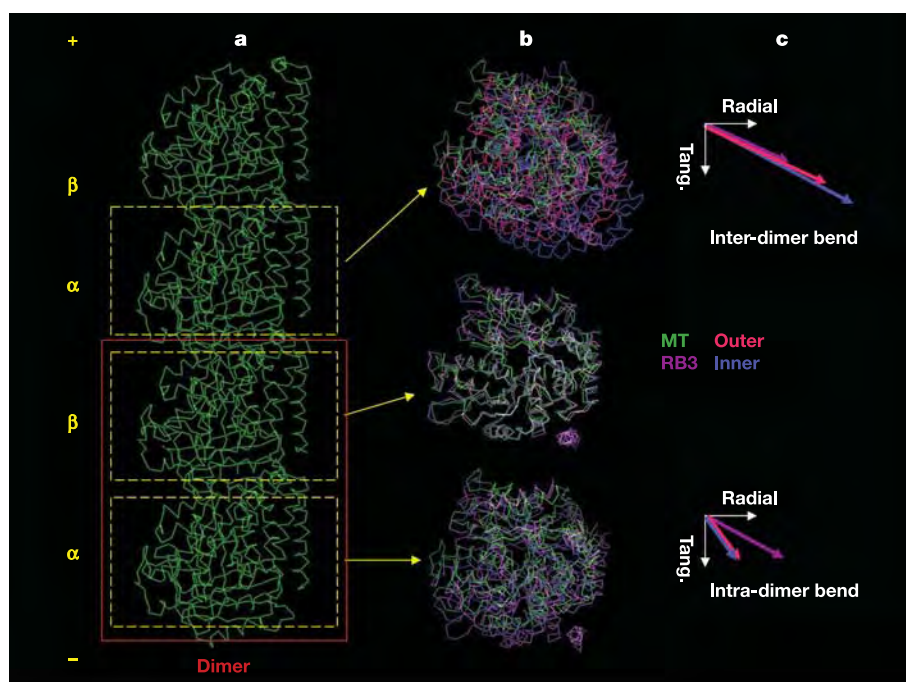


Figure 2 | Intra-dimer and inter-dimer bends in different tubulin polymers. **a**, Two dimers in a microtubule (plus-end at the top). The red box marks the lower dimer. The dashed yellow boxes indicate the monomers shown in the end-on views in **b**. **b**, The microtubule (green), RB3-bound structure (violet), outer layer (orange-red) and inner layer (blue) of the GDP

tubes were aligned on the first β -subunit. The bottom superposition shows the displacements due to the intra-dimer bending, and the top shows the displacements due to the inter-dimer bending. **c**, Relative magnitude (arrow length) and the radial and tangential (tang.) components of each bend.

monomers is not exactly that of either of the existing crystal structures, docking was slightly optimized when we used the β -tubulin structure from the RB3-bound model⁷ and the α -tubulin structure from the electron crystallography study⁵, indicating the possibility of two distinct monomer conformations.

The extent and direction of bending between tubulin monomers in this GDP state are similar but yet distinctive from that seen in the structure of the RB3/colchicine-tubulin complex (Fig. 2; Supplementary Fig. S4). The bending at the intra-dimer interface is smaller and more tangential. The inter-dimer and intra-dimer contacts are almost indistinguishable in RB3-tubulin, whereas in our structure the direction of inter-dimer bending is the same as in RB3-tubulin (and therefore slightly different from that within its dimer) but of significantly larger magnitude, which varies between the inner and outer layers. The small rearrangement seen at the intra-dimer interface in the RB3-tubulin structure might be due to the presence of colchicine at the site¹⁷. On the other hand, it is possible that the inter-dimer interface becomes locked into an 'intra-dimer-like' state by way of the RB3 α -helix that runs along the surface of both dimers⁷. Our study indicates that, irrespective of whether bound or unbound by a depolymerizer, the bending of the intra-dimer and inter-dimer interfaces in unconstrained, GDP-bound tubulin is incompatible with the formation of the canonical lateral contacts in microtubules. Although this explains why GDP-bound dimers or oligomers cannot be incorporated into the microtubule, it leaves open the question of how binding of GTP would result in the 'straightening' of protofilaments observed in microtubules.

Structure of GMPCPP-tubulin ribbons and tubes

The presence of GTP at the E-site would need to have an effect, both at the inter-dimer interface where it resides and at the intra-dimer interface 40 Å away, to straighten monomer contacts. One possibility is that binding of GTP straightens the intra-dimer and inter-dimer contacts to a point at which lateral contacts such as those in microtubules can form, and that further straightening occurs on closure of the polymer into a cylinder. This idea is suggested by cryoelectron microscopic (cryo-EM) images of microtubules with open, curved sheets at their growing ends⁸ that have been modelled to straighten mechanically as the microtubule closes¹⁸. The sheets are more often seen in conditions of fast growth, and it is not known whether hydrolysis occurs before sheet closure.

To reveal the curvature, structure and self-association of GTP-containing protofilaments we have studied the self-assembly of tubulin in the presence of GMPCPP. Previous studies showed that microtubules made of GMPCPP-bound tubulin have a slightly different axial repeat¹⁹, and when depolymerized by calcium they break down into curved protofilaments with markedly less curvature than those of GDP-containing microtubules²⁰. These results supported a model in which GTP hydrolysis increased the curvature of protofilaments, but the irregular nature of the peeled protofilaments precluded their characterization. Here we found that at low temperatures GMPCPP-bound tubulin forms helical ribbons reminiscent of the structures seen after the depolymerization of GMPCPP microtubules²⁰. The ribbons contain a few protofilaments early in the incubation process but grow to more than 20 protofilaments that close into a tube about 500 Å in diameter (Supplementary Fig. S5). Diffraction patterns from both small, open ribbons and larger, closed tubes show the same tubulin arrangement: head-to-tail association into protofilaments that interact laterally with the same stagger as in microtubules (Supplementary Fig. S6). We used cryo-EM and helical reconstruction to obtain a structure at 18 Å resolution of this GMPCPP state of tubulin (Fig. 3a). The structure (as predicted from the diffraction of ribbons and tubes) shows the presence of protofilament pairs (Fig. 3a and Supplementary Fig. S7). Docking of β -tubulin from zinc-sheets shows that the atomic model fits extremely well within our density (Fig. 3b). At this resolution α - and β -subunits are impossible to tell apart and the intra-dimer and

inter-dimer contacts are indistinguishable (notice that in this case both are in the same nucleotide state). The protofilaments are curved approximately radially, with the inside of our tubes corresponding to the outside of the microtubule. The radial bend between subunits is about 5° (Fig. 4a), significantly smaller than the intra-dimer bend of 12° in our GDP-bound structure. Thus, both interfaces straighten on binding GTP (GMPCPP).

We found that the lateral interactions between the two protofilaments in one pair are indistinguishable from those seen in microtubules, whereas the contacts between pairs use regions of tubulin that in microtubules define the external grooves between protofilaments (Fig. 4a). Thus, the alternate contacts have 'rolled out' by about 20 Å (about 60° rotation). Given this alternation of lateral interfaces it is remarkable that the lateral stagger is conserved (there is no longitudinal 'slippage'), indicating that this organization of tubulin might be related to the microtubule assembly process. This idea was supported by the direct conversion of ribbons into microtubules when the temperature was increased to 37°C (Fig. 4b, c). Closure into a microtubule requires the 'rolling back' of the alternate lateral contact and results in the straightening of protofilaments.

Our interpretation is that the GMPCPP ribbon structures are likely to correspond structurally to the curved ends of open sheets at the ends of growing microtubules⁸. At higher temperatures, closure into microtubules follows very closely the formation of the sheets; they are seen when the addition of subunits is significantly faster than hydrolysis, as in conditions of fast net polymerization⁸. If

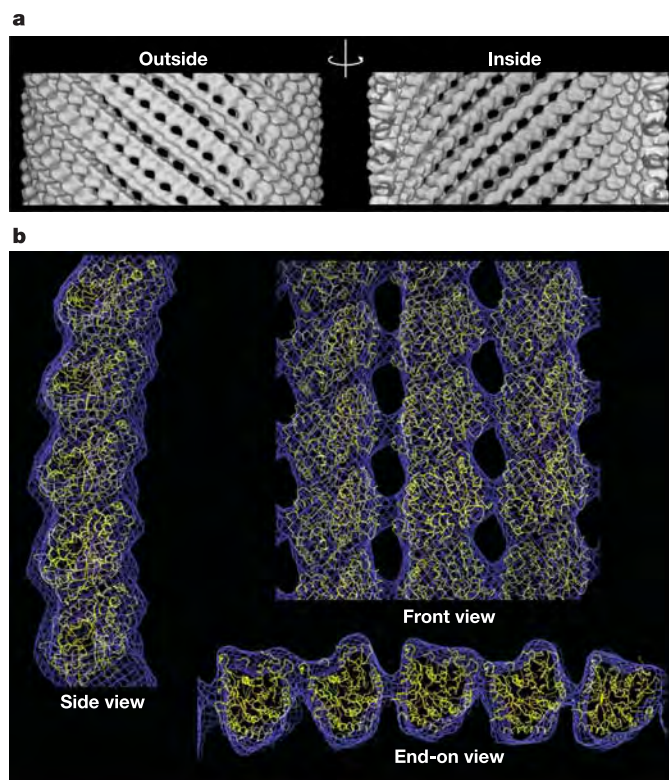


Figure 3 | Cryo-EM reconstruction of GMPCPP-tubulin tubes and docking of the crystallographic model. **a**, Three-dimensional densities of the GMPCPP-tubulin tubes. Protofilaments are associated in pairs. **b**, β -tubulin (1JFF) was manually docked into the density of the GMPCPP tube. The small outward curvature of the protofilaments is clearly seen in the side view on the left (right surface corresponds to the outside of the microtubule). The front view shows the lateral stagger between protofilaments, identical to that in microtubules. The end-on view shows more clearly the pairing of protofilaments. Within a pair the lateral contacts are indistinguishable from those in microtubules, but the lateral contact between pairs has been displaced towards the inside surface of the tube.

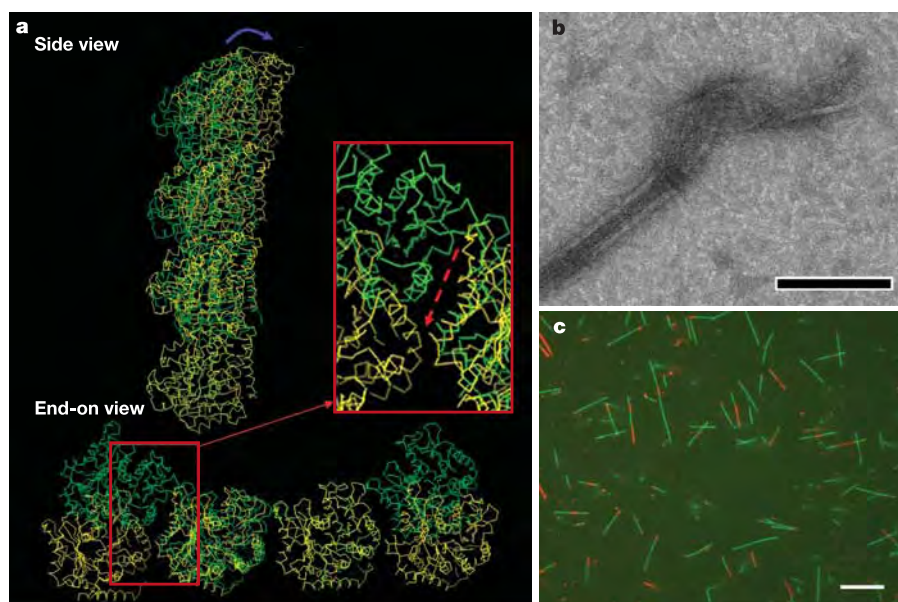


Figure 4 | GMPCPP tubes: comparison with and conversion into microtubules. **a**, GMPCPP tube (yellow) and microtubule (green). The side view illustrates radial bending (blue arrow); the end-on view shows how the lateral contact that results in the closure of microtubules is maintained within a protofilament pair but is displaced between pairs (red dashed arrow on inset). **b**, Direct conversion of GMPCPP tubes into microtubules at 37 °C

revealed by negative-stain electron microscopy. Scale bar, 100 nm.

c, Fluorescence microscopy of microtubules formed by mixing two populations of differentially labelled GMPCPP tubes before warming the solution, proving that conversion does not involve a depolymerization step. Scale bar, 10 μm.

GTP-tubulin self-associates at low temperatures, when closure is delayed or precluded, it rapidly hydrolyses GTP when longitudinal interactions are formed, and then curves into the GDP state. When hydrolysis is precluded with the use of a non-hydrolysable analogue, the straighter conformation is maintained and sheets (ribbons) grow that are able to close when the temperature is increased.

Structural pathway in the GTP-driven microtubule assembly

The present study shows that the GDP state of tubulin results in longitudinal self-association with two distinct kinks at the intra-dimer (GTP-containing) interface and the inter-dimer (GDP-containing) contact, the latter kink being more flexible and larger in magnitude. This indicates strongly that binding of GTP at the E-site will have an effect on the structure of tubulin, resulting in the formation of smaller and better-defined kinks between interacting dimers. Supporting experimental evidence comes from the visualization of peeling ends of calcium-depolymerized GMPCPP microtubules²⁰ and from the present structural study. The small tangential bend within the GDP-bound dimer might not be sufficient to inhibit binding to a growing microtubule end (major regions in lateral contacts are flexible loops that could accommodate a slightly different geometry). But the bend between GDP-tubulin dimers seems too large to permit the formation of lateral contacts, in agreement with the fact that GDP-bound tubulin cannot incorporate into microtubules. We propose that the binding of GTP at the E-site is likely to have three effects: first, to reduce the dimer–dimer bending by locally changing the conformation around the nucleotide at the interface; second, to straighten the dimer, a long-range allosteric change that could involve helix H7 and the following T7 loop in β -tubulin, which link the intra-dimer and inter-dimer interfaces; and third, to fine-tune the conformation of the monomer so as to strengthen lateral contacts. These three changes, or a subset of them, permit the partial straightening of protofilaments able to form lateral contacts that are otherwise inhibited in the more curved GDP state. Lateral association of tubulin into curved sheets would be followed by a distinct, final straightening process that closes the surface of the microtubule. Our study supports the separation of the straightening process into two

stages. It also provides a pseudo-atomic model of this sheet that illustrates a bimodal mechanism of lateral protofilament interaction preceding microtubule closure. Finally, our study proves that sheet closure does not require GTP hydrolysis.

METHODS

Formation and analysis of GDP-tubulin tubes. Double-layer tubes of GDP-bound tubulin were formed when partially subtilisin-cleaved tubulin was bound to GDP and incubated at 37 °C for a few hours in the presence of high concentrations of manganese. Frozen-hydrated helical tubes were then imaged by cryo-EM using a 200 keV, field emission gun (FEG) electron microscope. The images of individual tubes were classified into distinct helical families containing different numbers of subunits per turn. The 24/32 family was selected for further analysis and reconstruction using an iterative Fourier Bessel algorithm to determine the relative orientation of 19 tube images. Independent reconstructions for the inner and outer layers were then produced by Fourier integration, using data of up to 12-Å resolution. Details are provided in the Supplementary Information.

Formation and analysis of GMPCPP-tubulin tubes. Helical ribbons and tubes of GMPCPP-bound tubulin were obtained by incubating tubulin with the nucleotide at low temperatures (4–15 °C) for up to 4 h in the presence of a high concentration of magnesium ions (8–30 mM). Frozen-hydrated helical tubes were imaged by cryo-EM. The images of two tubes corresponding to the same helical family were analysed using traditional helical methods to obtain a reconstruction at 18-Å resolution.

Details regarding the formation and analysis of GMPCPP-tubulin tubes, the docking of atomic models in the reconstructions, and tracking of GMPCPP-tubulin tube conversion into microtubules using fluorescence microscopy are provided in the Supplementary Information.

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Author Information The density maps have been deposited at the Macromolecular Structure Database under accession numbers EMD-1129 (GDP-tube, inner layer), EMD-1130 (GDP-tube, outer layer), and EMD-1131 (GMPCPP-tube). Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to E.N. (enogales@lbl.gov).

LETTERS

Widespread magma oceans on asteroidal bodies in the early Solar System

Richard C. Greenwood¹, Ian A. Franchi¹, Albert Jambon² & Paul C. Buchanan³

Immediately following the formation of the Solar System, small planetary bodies accreted¹, some of which melted to produce igneous rocks^{2,3}. Over a longer timescale (15–33 Myr), the inner planets grew by incorporation of these smaller objects^{4,5} through collisions. Processes operating on such asteroids strongly influenced the final composition of these planets⁴, including Earth⁵. Currently there is little agreement about the nature of asteroidal igneous activity: proposals range from small-scale melting, to near total fusion and the formation of deep magma oceans². Here we report a study of oxygen isotopes in two basaltic meteorite suites, the HEDs (howardites, eucrites and diogenites, which are thought to sample the asteroid 4 Vesta⁶) and the angrites (from an unidentified asteroidal source). Our results demonstrate that these meteorite suites formed during early, global-scale melting (≥ 50 per cent) events. We show that magma oceans were present on all the differentiated Solar System bodies so far sampled. Magma oceans produced compositionally layered planetesimals; the modification of such bodies before incorporation into larger objects can explain some anomalous planetary features, such as Earth's high Mg/Si ratio.

Oxygen isotope analyses were undertaken by laser-assisted fluorination (Supplementary Methods). All major HED rock types (Fig. 1) and a representative suite of five angrite samples were analysed (full results are given in Supplementary Table 1). From the results it is clear that the HEDs and angrites formed from two isotopically distinct reservoirs (Fig. 1). The results do not support the $\Delta^{17}\text{O}$ value of -0.23 previously obtained for Angra dos Reis⁷, giving it an isotopic composition indistinguishable from the HEDs. ($\Delta^{17}\text{O}$ is defined in Supplementary Methods.) Using two distinct subsamples, we found that Angra dos Reis has a $\Delta^{17}\text{O}$ value of -0.080 ± 0.009 and plots on the angrite fractionation line (AFL) along with the other angrites studied.

The $\delta^{18}\text{O}$ variation displayed by the HEDs shows strong mineralogical control ($\delta^{18}\text{O}$ is defined in Supplementary Methods), with ^{18}O -rich values for eucrites reflecting high plagioclase contents, whereas ^{18}O -poor values for diogenites result from their orthopyroxene-rich mineralogy. Polymict breccias have major element compositions intermediate between diogenites and eucrites, being physical mixtures of these two end-members². This is also reflected in their oxygen isotope variation, with most samples plotting between the diogenites and eucrites (Fig. 1). Two exceptions are Pasamonte and Bholghatti, which plot above and below the eucrite fractionation line (EFL), respectively (Fig. 1). The deviation of Bholghatti is consistent with the presence of 1.3% carbonaceous chondrite material, similar to the C2 clasts previously identified in this howardite⁸. The polymict eucrite Pasamonte has a less negative $\Delta^{17}\text{O}$ value than the EFL, reflecting the presence of extraneous material with a relatively high $\Delta^{17}\text{O}$ value. Pasamonte is known to be contaminated by ejecta material, which siderophile element data

suggest was either H-group or CI chondrite⁹. If this material was exclusively ordinary chondrite-like, the isotopic composition of Pasamonte is consistent with a mixture of basaltic eucrite plus 3–3.5% of this non-indigenous component.

Basaltic eucrites have been subdivided into three compositional groups: (1) main group eucrites, (2) Nuevo Laredo trend eucrites, and (3) Stannern trend eucrites². We have analysed one sample from both the Nuevo Laredo (Lakangaon) and Stannern (Stannern) trends. These are isotopically indistinguishable from the other eucrites studied (Supplementary Table 1), and indicate that the equilibration of oxygen isotopes pre-dates the evolution of specific HED lithologies.

Various models have been proposed to account for the genesis of the HEDs. Early schemes invoked fractional crystallization, with the diogenites representing cumulates and the eucrites forming from the residual liquid¹⁰. Problems in reproducing the bulk composition of eucrites by this process led to the suggestion that they formed by low pressure partial melting (15–20%)¹¹. Total melting of the parent body has also been proposed, with subsequent core segregation and crystallization of the molten mantle to produce the diogenite-eucrite sequence¹². The merits of these models can be assessed by examining the conditions that led to oxygen isotopic homogenization in the HED parent body.

No group of chondritic meteorites has an oxygen isotope composition matching that of the HEDs. Consequently, proposed models for the bulk composition of the HED parent body generally invoke mixtures of more primitive components, such as 70% H chondrite: 30% CM chondrite¹³, or 70% L chondrite: 30% CV chondrite¹². Formation from pre-existing bodies is consistent with models indicating that early-formed planetesimals experienced significant collisional evolution¹⁴. The structure and grain size of the HED parent body immediately after formation is unknown, but may have resembled a 'rubble pile'¹⁵ comprising kilometre-sized blocks mixed with finer material. Formation from a mix of distinct primitive materials, such as, carbonaceous and ordinary chondrites, means that it must originally have been heterogeneous with respect to oxygen isotopes. However, the data presented here shows that the source of the HEDs had a uniform $\Delta^{17}\text{O}$ value of -0.239 ± 0.007 and hence must have undergone a homogenization event after initial accretion.

It is well established that the HED suite is extremely old, being not much younger than the age of the Solar System². Thus, the eucrite Asuka 881394 yields an age of $4,563.2 \pm 0.6$ Myr (ref. 3), only 4 Myr younger than that obtained from calcium and aluminium-rich inclusions (CAIs) in the meteorite Efremovka¹⁶ (CAI ages are widely accepted as dating Solar System formation). Mn–Cr dating indicates that the HED parent body formed and differentiated only 2.4 ± 0.9 Myr after CAI formation, thus coinciding with the final phase of chondrule formation¹. Hf–W dating also suggests that the

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HED parent body formed early, with core separation at 3 ± 1.4 Myr after CAI formation, followed by mantle differentiation 0.9 ± 0.3 Myr later⁴. These relationships suggest that parent body formation and differentiation occurred on a very short timescale, and constrain any model for the oxygen isotope evolution of the HED suite.

These time constraints exclude the possibility that oxygen isotopic equilibration could have occurred at subsolidus temperatures. Even if the parent body had formed at the same time as CAIs, then been heated instantaneously to $1,100^\circ\text{C}$ and remained at that temperature for 2.4 Myr, the diffusion distance (x) in olivine^{17,18} would only have been of the order of 0.4 mm. (Here $x = \sqrt{Dt}$, where D is the solid state diffusion coefficient and t is time.) This indicates that at the onset of partial melting ($1,170^\circ\text{C}$), the source region would have been heterogeneous with respect to oxygen isotopes. Partial melting of an unequilibrated source would have led to the formation of eucrites and diogenites with distinct isotopic compositions. Modelling suggests that differences in $\Delta^{17}\text{O}$ of over 0.2‰ between eucrites and diogenites would be produced by such a process (Supplementary Information). The fact that diogenites and eucrites both fall on the same mass fractionation line poses a significant difficulty for models

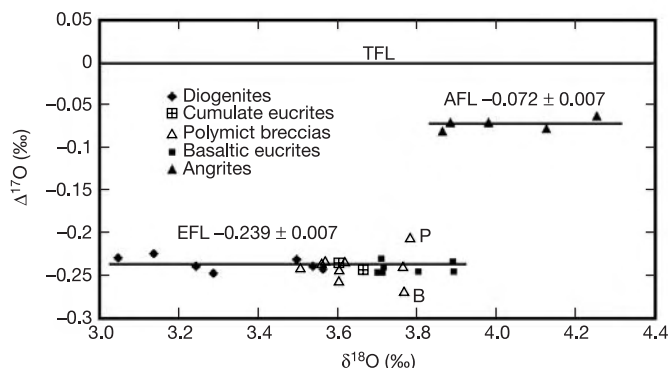


Figure 1 | Oxygen isotope variation diagram for HEDs and angrites.

Basaltic eucrites are fine- to medium-grained, commonly brecciated rocks composed predominantly of pigeonite and plagioclase ($\sim 70\text{--}92\%$ anorthite; $\sim \text{An}_{70\text{--}92}$)². Cumulate eucrites are coarse-grained gabbros that are mineralogically similar to basaltic eucrites, but with more calcic plagioclase ($\text{An}_{91\text{--}95}$). Diogenites are coarse-grained cumulates typically comprising 84–100% orthopyroxene. Polymict breccias (including howardites) consist of lithic and mineral clasts predominantly derived from various types of eucrites and diogenites². Angrites are unique among Solar System basalts in being the most alkali-depleted and silica-undersaturated². Angrites have very distinct mineralogies, comprising varying amounts of Al-Ti-rich diopside, anorthite, Ca-rich olivine, kirschsteinite and a variety of minor phases. In contrast to HEDs, angrites show little evidence of brecciation and often contain vesicles. On a $\Delta^{17}\text{O}$ versus $\delta^{18}\text{O}$ diagram, samples formed from a homogeneous reservoir that subsequently fractionated by mass-dependent processes plot along horizontal lines. Silicate minerals on Earth have isotopic compositions consistent with mass-dependent fractionation from a single reservoir²⁸, and define the terrestrial fractionation line (TFL). All five analysed angrites define a second horizontal line, the angrite fractionation line (AFL), with a mean $\Delta^{17}\text{O}$ value of -0.072 ± 0.007 (1σ). HED samples display greater $\Delta^{17}\text{O}$ variation than either the angrites or terrestrial silicates (Supplementary Table 1). However, HED polymict breccias are known to contain extraneous material, such as carbonaceous chondrite clasts⁸. If polymict breccias are excluded, the remaining HED samples ($n = 16$) show limited $\Delta^{17}\text{O}$ variation and define a single eucrite fractionation line (EFL) with a mean $\Delta^{17}\text{O}$ value of -0.239 ± 0.007 . Our $\Delta^{17}\text{O}$ value for the HED suite differs slightly from the previously determined value of -0.219 (ref. 29). This small discrepancy may reflect calibration differences and the fact that the previous study included meteorite finds, which have the potential to be terrestrially contaminated. The value for the EFL determined in the present study is based only on meteorite falls. P, Pasamonte; B, Bholghatti matrix sample.

invoking low degrees of partial melting to explain the genesis of the HED suite¹¹.

The geochemical evidence that the HED suite evolved after core formation¹² provides an important constraint when attempting to define the conditions under which the source region become isotopically homogeneous. It has been demonstrated that core separation requires a minimum of 50% melting of the silicate fraction¹⁹. At this level of partial melting, the mantle of the HED parent body would have consisted of convecting melt with entrained olivines and minor pyroxene. Although the melt will be well-mixed owing to convection, the olivine crystals may still be unequilibrated. Complete homogenization of the source would involve diffusive exchange between the olivines and enclosing melt. The scale of homogenization during 50% partial melting ($1,450^\circ\text{C}$) of a potential HED source composition¹² can be estimated from the diffusion time ($t = x^2/D$) for a 1-cm-diameter olivine crystal (x being the radius and D the diffusion coefficient¹⁷). This olivine size was chosen to match that found in pallasite meteorites². At $1,450^\circ\text{C}$, the timescales involved are of the order of 400,000 yr. In comparison, if melting took place at $1,350^\circ\text{C}$ (35% melting), oxygen isotope homogenization would require at least 2.6 Myr, which is too long on the basis of evidence from Hf-W isotopes⁴. The oxygen isotope data for the HEDs are therefore consistent with a model involving a minimum of 50% silicate partial melting, as required for effective separation of a Fe-Ni-S liquid to form the core. This scale of melting would correspond to a crystal-free melt layer of approximately 50 km depth.

Angrite samples plot on a well-defined mass fractionation line (Fig. 1), indicating that their parent body also underwent an early, global-scale oxygen isotope homogenization event. The angrites are equally as old as the HEDs, with D'Orbigny giving an age of $4,562.9 \pm 0.6$ Myr (ref. 20), that is, 4.3 Myr after CAI formation. Angrites are compositionally distinct from the HEDs, being critically silica undersaturated, whereas HEDs are hypersthene normative². Experimental work indicates that both HED-like and angrite-like melts can be produced from similar chondritic precursors by varying the oxygen fugacity (f_{O_2}) during melting²¹. Angrite-like liquids form under oxidizing conditions (with f_{O_2} ranging from IW + 1 to IW + 2, where IW indicates the iron-wüstite buffer) and eucritic liquids form under more reducing conditions ($f_{\text{O}_2} = \text{IW}-1$). In theory, both types of melts could have formed on the same asteroid if source conditions were locally variable. However, the oxygen isotope evidence presented here demonstrates that angrites and HEDs are the products of melting on two distinct parent bodies. In addition, both parent bodies had distinct post-formational histories,

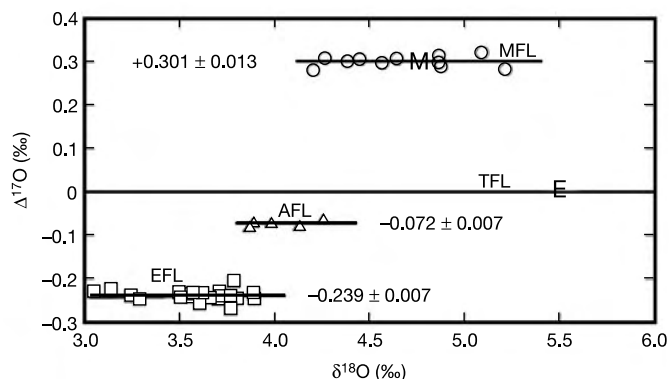


Figure 2 | Mass fractionation lines for Mars, Earth, Moon, Vesta and the angrite parent body. MFL, Mars fractionation line³⁰; TFL, terrestrial fractionation line; AFL, angrite fractionation line; EFL, eucrite fractionation line (Vesta). Data for the MFL from ref. 30. M, average mantle composition of Mars³⁰; E, average terrestrial upper-mantle composition²⁴.

with the HEDs being extensively modified by later impacts, whereas angrites show little evidence of brecciation.

We have suggested above that homogenization of oxygen isotopes was a consequence of high degrees of partial melting. One means of assessing this proposal is to look at the oxygen isotope variation in meteorite groups that experienced lower degrees of partial melting. Acapulcoites and lodranites formed by <1% to slightly greater than 20% partial melting of a single parent body²². Their $\Delta^{17}\text{O}$ values (-0.99‰ to -1.49‰)⁷ show a significantly greater range than seen among the HEDs or angrites, indicating that primary heterogeneities were preserved at low degrees of partial melting. Ureilites represent the residue formed when a 20–30% melt fraction was removed from a primitive chondritic precursor²³. Ureilites display major oxygen isotope variation, scattering about the slope-1 line defined by primitive carbonaceous chondrites⁷, again demonstrating that relatively low degrees of melting are insufficient to cause oxygen isotope homogenization.

The terrestrial planets and differentiated asteroids from which we have samples plot as separate, well-defined mass fractionation lines on a $\Delta^{17}\text{O}$ versus $\delta^{18}\text{O}$ diagram (Fig. 2). This demonstrates that each formed from a unique mix of precursor materials²⁴ and, following accretion, experienced at least one major phase of oxygen isotope homogenization. Global-scale homogenization of oxygen isotopes must have been a consequence of the same event that segregated the differentiated planets and asteroids into an Fe–Ni core²⁵ and silicate-rich mantle and crust. For the larger bodies (Earth, Moon and Mars), there is general agreement that core–mantle segregation occurred when the planet underwent almost total melting, leading to the formation of a magma ocean²⁵. Although similar models have been advanced for asteroids¹², there has been far less consensus about the importance of such magma oceans in the development of these smaller, differentiated bodies²⁶. The results of the present study clearly demonstrate that high levels of partial melting were attained on asteroid-sized bodies in the early Solar System.

Collisions between bodies in the early Solar System did not always result in accretion—significant erosion of their outer silicate portions also occurred¹⁴. Such reworking of the outer layers of differentiated asteroids may have important implications for the bulk composition of the planets formed by their accretion. Compositional layering is an inherent feature of asteroids that underwent a magma ocean stage, so that loss of their outermost Fe- and Si-rich crustal layers would increase the Mg/Si ratio of the residual remnant. The anomalously high Mg/Si ratio of Earth compared to chondrites may therefore result from collisional modification of the differentiated planetesimals from which it formed, rather than requiring sequestration of Si in the core, or the need to invoke an as-yet unsampled precursor material²⁴. The evidence presented here suggests that global-scale melting of differentiated asteroids was a major process in the early Solar System. Later modification of these bodies during the planet-building phase may have been a significantly more important process than has yet been considered. Imaging of the β Pictoris system suggests that such collisional reprocessing may be a widespread feature of planetary systems²⁷.

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High-frequency acoustic waves are not sufficient to heat the solar chromosphere

Astrid Fossum¹ & Mats Carlsson¹

One of the main unanswered questions in solar physics is why the Sun's outer atmosphere is hotter than its surface. Theory predicts abundant production of high-frequency (10–50 mHz) acoustic waves in subsurface layers of the Sun¹, and such waves are believed by many to constitute the dominant heating mechanism of the chromosphere (the lower part of the outer solar atmosphere) in non-magnetic regions^{2–4}. Such high-frequency waves are difficult to detect because of high-frequency disturbances in Earth's atmosphere (seeing) and other factors. Here we report the detection of high-frequency waves, and we use numerical simulations to show that the acoustic energy flux of these waves is too low, by a factor of at least ten, to balance the radiative losses in the solar chromosphere. Acoustic waves therefore cannot constitute the dominant heating mechanism of the solar chromosphere.

The Transition Region and Coronal Explorer⁵ (TRACE) spacecraft observes the Sun in ultraviolet and extreme-ultraviolet wavelengths. The passbands at 1,700 Å and 1,600 Å sample the upper photosphere and the temperature minimum region, respectively; the average

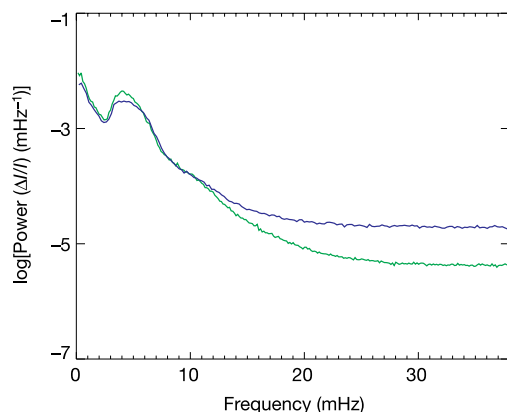


Figure 1 | Power of observed 1,600 Å TRACE relative intensity fluctuations. A special TRACE program was run, consisting of a continuous sequence of 1,600 and 1,700 Å images with a fixed cadence of 13 s (Nyquist frequency thus 38 mHz) and a higher-quality JPEG compression than the standard programs. After dark current subtraction, flat fielding and rigid alignment, a binning of 2 × 2 pixels was performed and the binned pixels were divided into categories using standard procedure²⁰. In this context we are only interested in the inter-network pixels, as we are studying the heating in non-magnetic areas. All pixels brighter than three standard deviations above the average brightness are thus discarded, and we are left with 18,684 and 15,684 pixels for the two days. We Fourier-transform the time series of each pixel and calculate the average over the pixels. The figure shows the one-sided power of observed 1,600 Å TRACE relative intensity fluctuations ($\Delta I/I$) on a logarithmic scale from 28 May 2003 (blue line) and 1 June 2003 (green line). The 1,700 Å results are similar.

formation height of the former is 360 km, and that of the latter is 430 km (ref. 6). These two regions are above the heights where most of the photospheric radiative damping of waves takes place, and together comprise the ideal region in which to attempt to determine the acoustic energy flux available for chromospheric heating. We have TRACE observations from two days in 2003. The power spectra for the intensity fluctuations in the 1,600 Å band are displayed in Fig. 1. We see that the observed power peaks at 3–5 mHz, which corresponds to a mixture of photospheric five-minute oscillations and chromospheric three-minute oscillations. Above 25–28 mHz, the TRACE intensity power plots show pure noise as they flatten out at a constant level.

Using numerical simulations, we can calculate the TRACE response to a given acoustic energy flux at 400 km height and thus determine whether the waves observed carry enough power to heat the chromosphere.

The numerical simulations used in this project were done with the radiation hydrodynamics code RADYN^{7–9}. The one-dimensional equations of mass, momentum, energy and charge conservation are solved, together with the non-local thermodynamic equilibrium (non-LTE) radiative transfer and population rate equations,

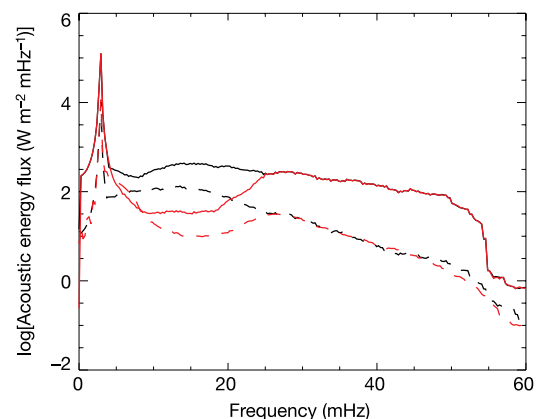


Figure 2 | Power of the acoustic energy fluxes in the simulations. The acoustic energy flux is taken as $v^2 \rho C_s$, where v is the velocity, ρ is the density and C_s is the sound speed. At the bottom boundary, the energy flux from run one (solid black line) peaks at 3.3 mHz and has a high-frequency maximum at about 15 mHz with a slow decline up to 50 mHz. Above that there is no significant power. At 400 km height (dashed black line), the flux has decreased in power. The shape of the power from the second run at the bottom of the domain (solid red line) and at 400 km height (dashed red line) differs from the first in the region 5–25 mHz. The third run is just the same as the second run but with the velocity scaled by 0.5 and thus with a power scaled by 0.25.

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Table 1 | Total energy fluxes

	Run 1	Run 2	Run 3	Radiative energy losses ¹⁵	Determined from TRACE
Piston	10.829	5.657	1.414	4.300	438
400 km	2.213	849	212		

All numbers are given in W m^{-2} and are fluxes integrated over 5–50 mHz. The determination from TRACE observations reported here is an upper limit based on observed intensity variations in the range 5–28 mHz and the noise level in the range 28–50 mHz. The high-frequency acoustic energy flux has to balance the radiative energy losses¹⁵ in order to heat the chromosphere.

implicitly on an adaptive mesh¹⁰. The equations are solved simultaneously and implicitly to ensure self-consistency and stability in the presence of radiative energy transfer and stiff population rate equations. The effects of non-equilibrium ionization, excitation, and radiative energy exchange from several atomic species (H, He and Ca) on fluid motions and the effect of motion on the emitted radiation from these species are calculated. Continua from elements other than H, He and Ca are treated as background continua in LTE, using the Uppsala atmospheres program¹¹. The opacity within the TRACE 1,600 Å and 1,700 Å bands is dominated by bound-free opacity from neutral silicon and by a very large number of lines from various elements. We treat silicon as a minority ion, and solve the rate equations for silicon with the density and electron density as functions of height and time as given by the fully coupled solution. Line blanketing is included by sampling the opacity at 0.1 Å intervals in the TRACE passbands using tabulated opacity data¹² and including scattering with a scattering fraction from a two-level approximation with the collisional de-excitation calculated from approximate¹³ formulae.

We have made three simulations for this purpose. They have the same initial atmosphere: a constant flux atmosphere with the solar effective temperature of 5,780 K. The sound speed is approximately 7 km s^{-1} and the acoustic cut-off frequency is about 5 mHz. The upper boundary is a corona at 10^6 K at 10^4 km height with a transmitting boundary condition. Incident radiation from the corona is taken from observations¹⁴. Waves are driven through the atmosphere by prescribing the velocity as function of time at the bottom boundary (86 km below optical depth unity at 5,000 Å). In the first run, the given velocities at the lower boundary (piston velocities) were taken from a theoretical model for wave excitation (P. Ulmschneider, personal communication). The second

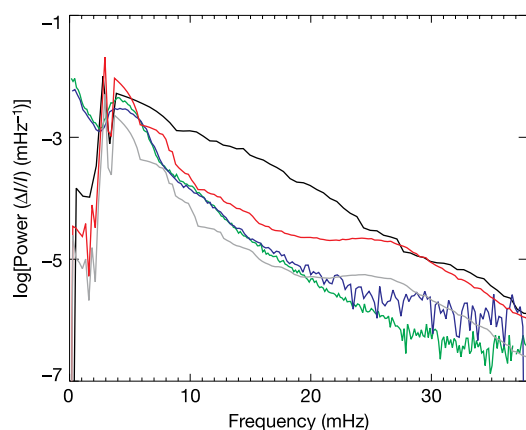


Figure 3 | Simulated and observed 1,600 Å TRACE intensity fluctuations. The observed power ($\Delta I/I$) has here been corrected for noise by subtracting white noise at a level equal to the minimum power level at the highest frequencies. The blue and green lines display the observations from 28 May and 1 June, respectively; the black, red and grey lines display the power from the three simulations. The power plots from the simulations are smoothed above 5 mHz. The 1,700 Å results are similar.

run was made using a similar piston but with lower power in the region 5–25 mHz. The third run had the piston velocities further lowered by a factor of two. The outcome of our numerical simulations is the intensity as a function of wavelength and time. This intensity is transformed into two signals equivalent to what we see through the TRACE 1,700 and 1,600 Å passbands by multiplying the intensity with the TRACE filter transmission functions and integrating over wavelength.

Figure 2 shows the power spectrum of the acoustic energy fluxes at the bottom of the computational domain and at 400 km height. The two runs differ in the region 5–25 mHz, but they both have a slow decline up to 50 mHz. Above that, there is no significant power. The total energy fluxes for the propagating waves (above the cut-off frequency of 5 mHz) at the bottom of the computational domain and at 400 km height are given in Table 1. According to the VAL3-C model¹⁵, the radiative energy losses in the chromosphere amount to $4,300 \text{ W m}^{-2}$. This is twice the amount that our energy flux from run one at 400 km can balance, and more than five times the amount of flux from run 2. This implies that the intensity fluctuations detected from authentic TRACE images have to be larger than the intensity fluctuations from our simulations for the high-frequency acoustic waves to provide enough power to heat the chromosphere.

Figure 3 shows the power of the intensity fluctuations from the three runs in addition to the observed TRACE intensities. Contrary to what is needed for the heating of the chromosphere, we see that the observed TRACE intensity fluctuations have less power than the intensity fluctuations from run one up to the Nyquist frequency of the observations at 38 mHz.

Using the simulations, we can estimate the energy flux that corresponds to the observed TRACE intensity fluctuations. This is shown in Fig. 4. The determined acoustic energy flux decreases with frequency and has no peak around 20 mHz. This is in contradiction to analytic results based on simplified descriptions of convection¹, but is consistent with large-scale numerical simulations of the convective generation of acoustic waves^{16,17}. The integrated energy flux up to where noise overwhelms the signal (around 28 mHz) is 346 W m^{-2} . Using the noise level as an upper limit to the real signal (dashed in Fig. 4), we get an upper limit to the acoustic energy flux in the range 5–50 mHz of 438 W m^{-2} (see Table 1), which is

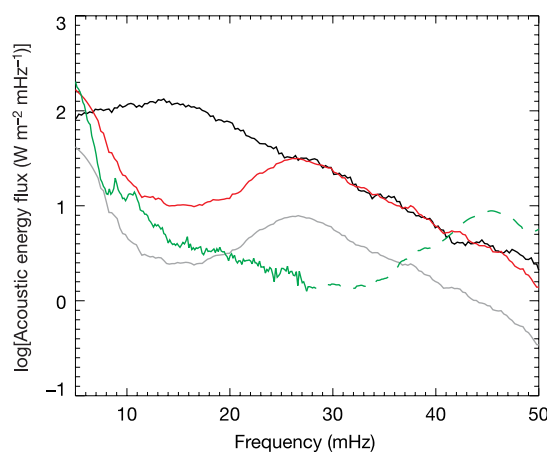


Figure 4 | Simulated and derived acoustic energy fluxes at a height of 400 km. We assume that the relationship between the intensity and the energy flux at 400 km is $(\Delta I/I)^2 = \alpha F^\beta$, where F is the energy flux and α and β are constants determined as functions of frequency from the three simulations. The black, red and grey lines display the energy fluxes from the three simulations, and the green line displays the derived energy flux from the TRACE observations of 1 June. The solid green line is drawn for the region where we have detected signal from TRACE, and the dashed line is giving the upper limit set by the noise.

about one-tenth of what is needed to account for the radiative losses.

We have determined the acoustic energy flux in the 5–28 mHz range, and set an upper limit to the flux up to 50 mHz. Even higher-frequency waves are unlikely to be important, given the strong radiative damping that these waves undergo in the upper photosphere¹⁸. The computational uncertainty is also judged to be insufficient to allow for high-frequency acoustic waves to have enough energy to heat the chromosphere; the calculated TRACE count rates from the simulations agree with the observed values to within 20%. We thus conclude that the acoustic energy flux is insufficient to balance the radiative losses in the non-magnetic solar chromosphere.

What then is the heating mechanism of the non-magnetic chromosphere? It is important to realize that the concept of a non-magnetic chromosphere is at best valid in the low chromosphere—in the middle to upper chromosphere, the magnetic fields have spread and fill the area. Even in the photosphere, most of the area may be filled with weak fields or with stronger fields with smaller filling factor¹⁹. Our results show that the emission from the middle and upper chromosphere must be balanced by processes related to the magnetic field. The heating of the lower chromosphere in ‘non-magnetic’ areas is more of a mystery. As shown, high-frequency waves can only make a small contribution to the heating, and magnetic fields are too weak or have too small a filling factor to be of importance. The lower chromosphere may be wholly dynamic in nature, with energy input from acoustic waves with periods of around three minutes.

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LETTERS

'Magic' nucleus ^{42}Si

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Nuclear shell structures—the distribution of the quantum states of individual protons and neutrons—provide one of our most important guides for understanding the stability of atomic nuclei. Nuclei with 'magic numbers' of protons and/or neutrons (corresponding to closed shells of strongly bound nucleons) are particularly stable^{1,2}. Whether the major shell closures and magic numbers change in very neutron-rich nuclei (potentially causing shape deformations) is a fundamental, and at present open, question^{3,4}. A unique opportunity to study these shell effects is offered by the ^{42}Si nucleus, which has 28 neutrons—a magic number in stable nuclei—and 14 protons. This nucleus has a 12-neutron excess over the heaviest stable silicon nuclide, and has only one neutron fewer than the heaviest silicon nuclide observed so far⁵. Here we report measurements of ^{42}Si and two neighbouring nuclei using a technique involving one- and two-nucleon knockout from beams of exotic nuclei^{6,7}. We present strong evidence for a well-developed proton subshell closure at $Z = 14$ (14 protons), the near degeneracy of two different ($s_{1/2}$ and $d_{3/2}$) proton orbits in the vicinity of ^{42}Si , and a nearly spherical shape for ^{42}Si .

The nuclide ^{42}Si has become the focus of particular interest in discussions of nuclear shell structure at the neutron drip line, which

is the locus of the heaviest nuclides in which all the neutrons are bound to the nucleus. The nuclide ^{42}Si is only one neutron lighter than the heaviest silicon nucleus yet observed⁵ and is close to the drip line, as illustrated in Fig. 1. The $N = 28$ (28 neutron) major shell closure is the lightest neutron shell closure caused by the spin-orbit force, which is responsible for all major shell closures in heavier nuclei^{1,2}. However, the increasing experimental difficulty of reaching the neutron drip line with increasing proton number means that this $N = 28$ case is the only such spin-orbit shell closure that can currently be examined at the neutron drip line. In fact, it has been predicted^{8–14} that this shell closure should be less well developed, or even collapse altogether, in ^{42}Si , resulting in a strongly deformed shape for this nucleus. Two recent experimental results—one a measurement¹⁵ of the β -decay half-life of ^{42}Si and the other the determination that ^{43}Si is particle-bound⁵—have been cited in support of this view. On the other hand, ^{42}Si , with 14 protons, is expected to have a good proton subshell closure and, if the $N = 28$ major shell closure is also maintained, then ^{42}Si should have a distinctive spectroscopy characteristic of a rigidly spherical shape, like the $N = 28$ isotone ^{48}Ca or the $N = 20$ nuclide ^{34}Si (ref. 16). Shell model calculations¹⁷ predict a situation between these two extremes: a weakly deformed shape for ^{42}Si in which the $N = 28$ shell

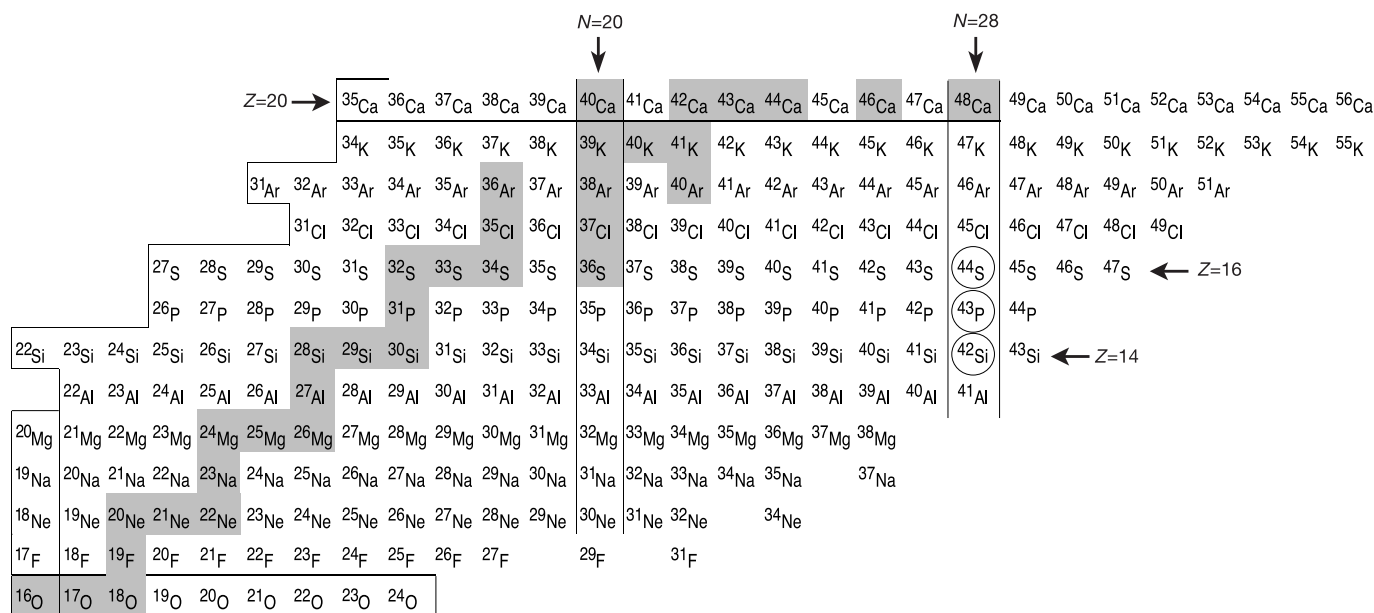


Figure 1 | A chart showing all particle-bound nuclides of the elements having $Z = 8$ –20. The stable nuclides are shaded. The nuclei studied

here— ^{42}Si , ^{43}P and ^{44}S —are circled. N , number of neutrons; Z , number of protons.

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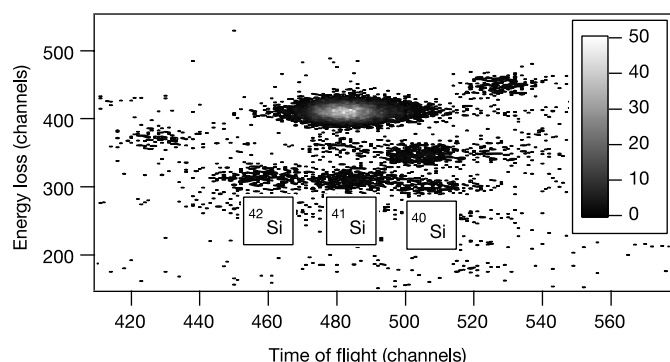


Figure 2 | Particle spectrum used to identify the two proton-knockout product ^{42}Si . Plotted are the energy loss in a plastic scintillator at the S800 spectrograph focal plane on the y axis, and the time of flight through the spectrograph on the x axis. The energy-loss signal groups ions of the same Z together, while the time of flight in conjunction with the magnetic rigidity selection of the spectrograph distinguishes ions according to their mass/charge ratio. The shade of grey represents the number of events detected in each bin, according to the scale displayed on the right hand side of the figure.

gap is narrower than in ^{48}Ca but in which the $Z = 14$ subshell closure prevents strong deformation.

In the present study, the nuclides ^{43}P and ^{42}Si were produced using respectively one- and two-proton knockout reactions with a beam of the exotic nucleus ^{44}S . In addition, a ^{46}Ar beam was used to perform spectroscopy of ^{44}S via the two-proton knockout reaction. The experiment was performed with the Coupled Cyclotron Facility (CCF) at the National Superconducting Cyclotron Laboratory (NSCL) at Michigan State University. The A1900 fragment separator¹⁸ was used to produce the exotic beams (which had kinetic energies of 98.6 MeV per nucleon and 98.1 MeV per nucleon for ^{44}S and ^{46}Ar , respectively), the S800 spectrograph¹⁹ to detect and identify the residues of the knockout reactions, and the SeGA array of segmented germanium detectors²⁰ to detect γ -rays from the residues. The knockout reactions took place in a beryllium target of thickness 375 mg cm^{-2} .

The extreme sensitivity in this experiment can be illustrated in the following way: the exotic ^{44}S and ^{46}Ar beams were produced via fragmentation reactions with a stable ^{48}Ca 'primary' beam. The primary beam current was 15 particle nanoamperes, or 3.4×10^{14} particles h^{-1} . From this, 1.5×10^6 particles of the ^{44}S exotic beam were produced per hour. Of the three residual nuclides studied here, the one with the smallest yield was ^{42}Si , which gave $1.6 \text{ counts h}^{-1}$. The particle spectrum used to identify ^{42}Si is shown in Fig. 2.

The γ -ray energy spectrum obtained in coincidence with the ^{43}P residues, following one-proton removal from ^{44}S , is shown in Fig. 3. The spectrum is shown in the rest frame of the residues; that is, the γ -ray energies are Doppler corrected from the laboratory frame. A single strong γ -ray transition appears in this ^{43}P γ -ray spectrum at $184 \pm 3 \text{ keV}$, the first γ -ray observed in this nucleus. The single-nucleon knockout reaction preferentially populates states that have a structure of a single nucleon hole in the beam nucleus⁶. The measured cross-sections for populating the two states of ^{43}P observed here—the ground state and the excited state de-excited by the 184 keV γ -ray—are large, so an interpretation of these two states as $d_{3/2}$ and $s_{1/2}$ single-proton states is justified. Their sum is $7.6(11) \text{ mb}$. This cross-section can be compared to theoretical cross-sections calculated for pure $d_{3/2}$ and $s_{1/2}$ single-proton states using the prescription described in refs 21 and 22. These theoretical cross-sections are 7.7 and 6.1 mb, respectively. An examination of the measured cross-section using γ -ray coincidences shows that the excited state accounts for $75 \pm 15\%$ of the cross-section. Furthermore, the 184 keV γ -ray probably connects the two states,

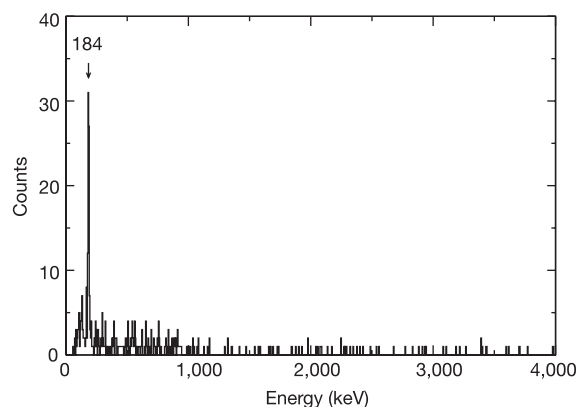


Figure 3 | The γ -ray energy spectrum in coincidence with ^{43}P residues. The γ -rays are Doppler-corrected so that the spectrum appears as in the rest frame of the residues. The γ -ray observed at $184 \pm 3 \text{ keV}$ and described in the text is labelled.

setting the energy splitting of the two orbits.

We performed a shell model calculation of the wavefunctions of the ground state and lowest excited state in ^{43}P that provides strong evidence for the identification of the excited state with the $d_{3/2}$ proton orbit and the ground state with the $s_{1/2}$ orbit. The calculations were carried out in the model space of $(0d_{5/2}, 1s_{1/2}, 0d_{3/2})$ for protons and $(0f_{7/2}, 1p_{3/2}, 0f_{5/2}, 1p_{1/2})$ for neutrons with an effective interaction used in ref. 23. The computer code OXBASH²⁴ was used to perform the shell model calculations. The one-proton knockout cross-sections were calculated from the wavefunctions using the approach described in refs 21 and 22. According to the calculation, the state corresponding to the $d_{3/2}$ proton orbit should have 72% of the total cross-section in the present one-proton knockout reaction. The share of the cross-section measured for the excited state, $75 \pm 15\%$, provides a strong argument for the identification of the excited state with the $d_{3/2}$ proton orbit and the ground state with the $s_{1/2}$ orbit. The near-degeneracy of these two proton orbits seen in the data (with a splitting of 184 keV) is also seen in the shell model calculation with a splitting of 1 keV. This theoretical result is consistent with the observed splitting, because shell-model calculations of this type are generally accurate to within 100–200 keV at best. The shell model fit of ref. 25 also predicted this near-degeneracy with a splitting of 100 keV.

A previous study of proton hole states populated in the potassium nuclides using the $^{X}\text{Ca}(d, ^3\text{He})$ reaction at low energy²⁶ provides some perspective for the ^{43}P result. The relative energies of the $d_{3/2}$, $s_{1/2}$ and $d_{5/2}$ proton orbits extracted¹⁶ from these data are shown in Fig. 4, as are the $Z = 8$ and $Z = 20$ major proton shell closures and the $Z = 14$ and 16 subshell closures. The energy gap between the $d_{3/2}$ and $s_{1/2}$ proton orbits decreases dramatically from more than 2 MeV in the $N = 20$ nuclide ^{39}K , where this gap forms the $Z = 16$ subshell closure, to approximately 300 keV in the $N = 28$ nuclide ^{47}K , collapsing the $Z = 16$ closure. Such shifts are observed throughout the periodic table, as demonstrated recently²⁷ in the tin nuclides and the $N = 82$ isotones. The separation between the $d_{5/2}$ and $s_{1/2}$ proton orbits remains large—averaging 5 MeV—throughout the K nuclides. The large energy gap between the $d_{5/2}$ proton orbit and the two higher-lying orbits forms a strong $Z = 14$ subshell closure, which is more robust than the $Z = 16$ closure. The present result shows that the $d_{3/2}$ and $s_{1/2}$ proton orbits maintain their near-degeneracy in ^{43}P . It is remarkable that the energy difference between the $d_{3/2}$ and $s_{1/2}$ proton orbits remains so stable along the $N = 28$ isotones from ^{47}K to ^{43}P (as illustrated in Fig. 4) whereas it changes so dramatically along the potassium isotopic chain.

Whereas the ^{43}P result provides strong evidence for the near-degeneracy of the $d_{3/2}$ and $s_{1/2}$ proton orbits, the cross-sections

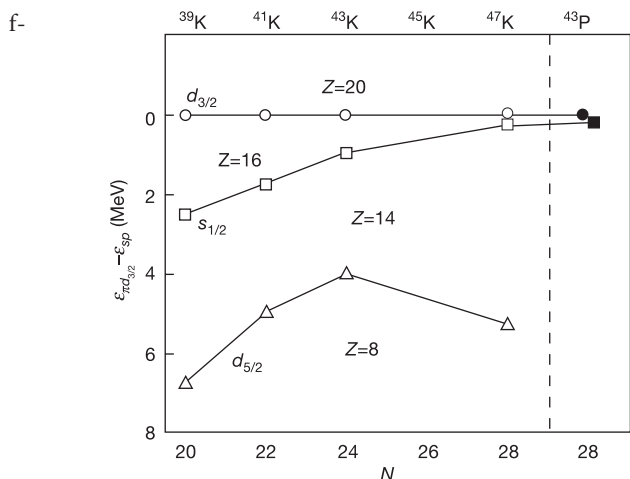


Figure 4 | Relative single proton energies. Shown are the relative energies of the $d_{3/2}$, $s_{1/2}$ and $d_{5/2}$ proton orbits in the K nuclides as extracted in ref. 14 from the data of ref. 23 (open symbols), and the relative energies of the $s_{1/2}$ and $d_{3/2}$ proton orbits in ^{43}P as determined in the present work (filled symbols). The energies are given relative to the energy of the $d_{3/2}$ proton orbit, $\epsilon_{\pi d_{3/2}}$, so that they are represented as the differences between the single proton energies ϵ_{sp} and $\epsilon_{\pi d_{3/2}}$.

or the two-proton knockout reactions $^{44}\text{S} \rightarrow ^{42}\text{Si}$ and $^{46}\text{Ar} \rightarrow ^{44}\text{S}$ provide evidence that the $Z = 14$ subshell closure that exists at ^{48}Ca persists near the neutron drip line in the vicinity of ^{42}Si . The inclusive cross-sections for these reactions (0.23(2) mb for $^{46}\text{Ar} \rightarrow ^{44}\text{S}$ and 0.12(2) mb for $^{44}\text{S} \rightarrow ^{42}\text{Si}$) are significantly smaller than those for the other two-proton knockout reactions measured to date⁷ (1.50(10) mb for $^{28}\text{Mg} \rightarrow ^{26}\text{Ne}$; 0.76(10) mb for $^{34}\text{Si} \rightarrow ^{32}\text{Mg}$; and 0.49(5) mb for $^{30}\text{Mg} \rightarrow ^{28}\text{Ne}$). The cross-section for the two-proton knockout reaction depends in part on the number of valence protons in the projectile nucleus—the smaller the number of valence protons, the smaller the cross-section is expected to be⁷. If the $Z = 14$ subshell closure persists to ^{42}Si , then the number of valence protons is small in ^{46}Ar (four) and even smaller in ^{44}S (two). Therefore, the decrease in cross-section from the $^{46}\text{Ar} \rightarrow ^{44}\text{S}$ reaction to the $^{44}\text{S} \rightarrow ^{42}\text{Si}$ reaction is qualitative evidence for the persistence of the $Z = 14$ shell closure at $N = 28$.

We can provide a quantitative basis for this two-proton knockout discussion using a calculation that combines the shell model calculations described above—which include the $Z = 14$ subshell closure—with eikonal reaction theory. The present calculation scheme²⁸ is more complete than that given in ref. 7. We calculate only the dominant stripping contribution to the two-nucleon removal cross-section. As the two-proton knockout reactions are from deeply-bound single-particle states, contributions from diffraction dissociation processes are assumed to be small. The shell model calculations for the two-proton amplitudes were obtained with a truncated space of $(0f_{7/2}, 1p_{3/2})$ for neutrons (in order to keep feasible matrix dimensions for OXBASH²⁴), but comparison with the full-space results for ^{46}Ar and ^{44}S showed that this truncation is adequate. In this calculation, the ground state of ^{42}Si is dominated (about 70%) by the closed-shell configuration of $(0d_{5/2})^6$ for protons and $(0f_{7/2})^8$ for neutrons and is therefore not well deformed, although it is not as spherical as ^{34}Si and ^{48}Ca .

The calculation yields inclusive two-proton knockout cross-sections of 0.36 mb and 0.17 mb for the $^{46}\text{Ar} \rightarrow ^{44}\text{S}$ and $^{44}\text{S} \rightarrow ^{42}\text{Si}$ reactions, respectively. These calculations, which include the $Z = 14$ subshell closure, reproduce the small magnitudes of the measured cross-sections (relative to those previously measured⁷); the reduction factors are similar to those of the single-proton knockout reactions. The decrease in cross-section from the $^{46}\text{Ar} \rightarrow ^{44}\text{S}$ reaction to the $^{44}\text{S} \rightarrow ^{42}\text{Si}$ reaction is also reproduced. A further schematic calculation in which the $Z = 14$ subshell closure is removed *ad hoc* yields

cross-sections that are much larger than the data, confirming that the small observed cross-sections imply the persistence of the closure near ^{42}Si .

As pointed out in ref. 17, the persistence of the strong $Z = 14$ subshell closure in ^{42}Si will prevent strong deformation and will result in a nearly spherical shape. The present results suggest that the short lifetime of ^{42}Si , reported in ref. 15, and the existence of ^{43}Si , reported in ref. 5, must now be explained without the strong deformation assumed by the authors of those reports.

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Triplet-singlet spin relaxation via nuclei in a double quantum dot

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The spin of a confined electron, when oriented originally in some direction, will lose memory of that orientation after some time. Physical mechanisms leading to this relaxation of spin memory typically involve either coupling of the electron spin to its orbital motion or to nuclear spins^{1–7}. Relaxation of confined electron spin has been previously measured only for Zeeman or exchange split spin states, where spin-orbit effects dominate relaxation^{8–10}; spin flips due to nuclei have been observed in optical spectroscopy studies¹¹. Using an isolated GaAs double quantum dot defined by electrostatic gates and direct time domain measurements, we investigate in detail spin relaxation for arbitrary splitting of spin states. Here we show that electron spin flips are dominated by nuclear interactions and are slowed by several orders of magnitude when a magnetic field of a few millitesla is applied. These results have significant implications for spin-based information processing¹².

The coupling of nuclear spins to electrons in low-dimensional semiconductors is known from optical and transport studies in quantum Hall systems to yield rich physical effects and provide

new probes of the relatively isolated quantum system of nuclear spins in solids^{13–16}. Confined electrons interacting with relatively few nuclei are particularly sensitive to hyperfine coupling. This can lead to dramatic effects such as tunnelling currents that slowly oscillate in time and electrical control and readout of nuclear polarization^{17,18}. Here we show that the interaction between single electrons confined in quantum dots with ensembles of lattice nuclei can dominate electron spin relaxation.

We use high-frequency pulsed gates to measure spin relaxation in a GaAs double quantum dot (Fig. 1a). Measurements are performed near the (1,1) to (0,2) charge transition, where (n,m) denotes the absolute number of electrons on the left and right dots. In the (0,2) configuration, the two electrons form a spin singlet to avoid the large Pauli exclusion energy cost ($0.4\text{ meV} \gg kT \approx 10\text{ }\mu\text{eV}$) of occupying an excited orbital state^{19,20}. In the separated (1,1) configuration, the two electrons may occupy any spin state. That is, apart from any Zeeman energy ($\sim 2.5\text{ }\mu\text{eV}$ at 100 mT), the singlet, (1,1)S, and three triplets, (1,1)T₊, (1,1)T₀, and (1,1)T_– ($m_s = -1, 0, 1$ respectively), are effectively degenerate, given the weak interdot coupling to which the system is tuned.

Spin relaxation is measured by preparing an unpolarized mixture of (1,1) states and monitoring the probability of transition to (0,2)S

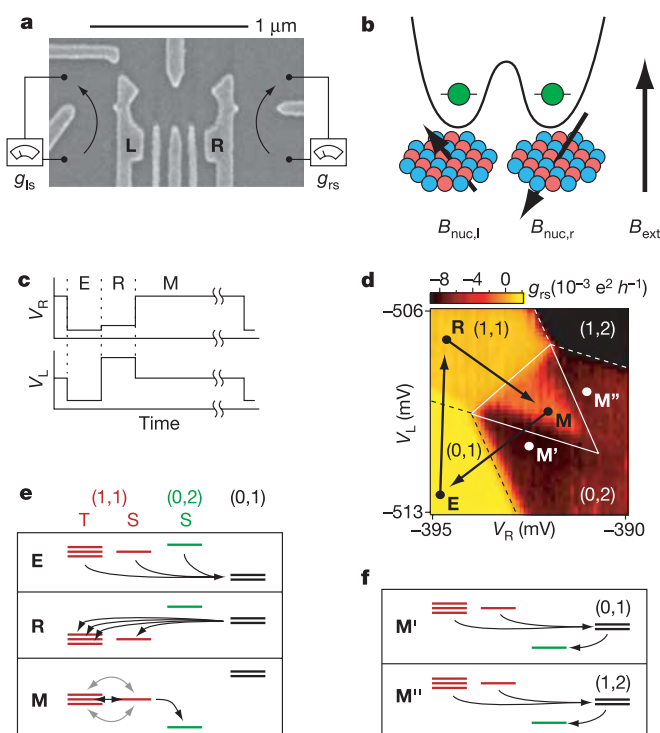


Figure 1 | Spin-selective tunnelling in a double quantum dot. **a**, Micrograph of a device similar to the one measured. Metal gates deplete a two-dimensional electron gas 100 nm below the surface, with density $2 \times 10^{11}\text{ cm}^{-2}$. A double dot is defined between gates L and R. Electrons tunnel between the dots and to conducting leads. Conductances g_{Ls} and g_{Rs} of the left and right QPCs reflect average occupation of each dot. **b**, In (1,1), spatially separated electrons feel different effective fields from hyperfine interaction with the local Ga and As nuclei, plus a uniform external field. **c**, Voltage pulses on gates L and R cycle through three configurations: empty (E), reset (R) and measure (M). **d**, Right sensor conductance g_{Rs} as a function of direct-current voltages on the same two gates around the (1,1) to (0,2) transition, with pulse displacements shown by points E, R, and M. Dashed lines outline the (0,1), (1,1), (0,2), and (1,2) charge state plateaus during step M. Inside the solid-outlined 'pulse triangle', the ground state is (0,2), but higher sensor conductance indicates partially blocked tunnelling. A plane is subtracted from the raw data to remove direct gate-QPC coupling. **e**, Energetics of the pulse sequence. In (0,2), only the singlet is accessible, whereas in (1,1), singlet and triplet are degenerate. (0,1) and (1,2) are spin-1/2 doublets. Step E empties the second electron, then R loads a new electron into the left dot, occupying all four (1,1) states equally. At M, (0,2)S is the ground state, but only (1,1)S and the $m_s = 0$ triplet (1,1)T₀ can tunnel. Mixing of (1,1)T₊ and (1,1)T_– with the singlet is weak away from zero field, so their tunnelling is blocked. **f**, At M', (0,1) has lower energy than (1,1) and provides an alternate, spin-independent path to (0,2). At M'', (1,2) provides this alternate path.

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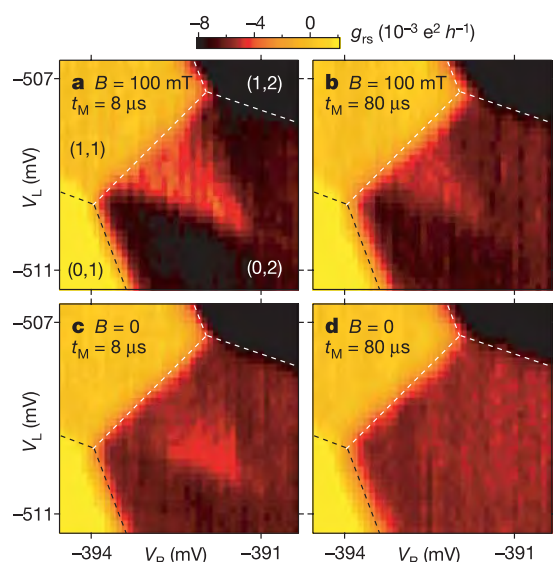


Figure 2 | Dependence of the occupancy of the (1,1) state on measurement time, t_M , and external field, B . **a**, Charge sensor conductance, g_{rs} , as a function of V_L and V_R with short pulses ($t_M = 8 \mu s$) at $B = 100$ mT. Large average occupation of (1,1) is seen throughout the pulse triangle. Near the triangle edges, thermally activated tunnelling to the leads allows fast relaxation to (0,2), (see Fig. 1f). **b**, For longer pulses ($t_M = 80 \mu s$), thermally relaxed triangle edges expand towards the centre of the triangle. **c**, At $B = 0$, the (1,1) occupation is extinguished at low detuning (near the (1,1)-(0,2) charge transition) as tunnelling to (0,2) becomes possible from the (1,1) T_+ and (1,1) T_- states. **d**, Combine these two effects at zero field with long pulses, and no residual (1,1) occupation is seen, indicating complete relaxation to (0,2).

after the latter is made lower in energy by changing the electrostatic gate configuration. The different local environments acting on the two spins cause the two-electron spin state to evolve in time, and only if this spin state passes near (1,1)S is a transition to (0,2)S allowed. The average occupancy of the left dot, which reflects the probability of this transition, is monitored using local quantum point contact (QPC) charge sensors¹⁹. Conductances g_{ls} and g_{rs} of the left and right sensors change by several per cent when an electron enters the dot nearest the sensor^{21–24}.

The energy levels of each dot were controlled by voltage pulses on gates L and R, as shown in Fig. 1c (ref. 19, and see also Supplementary Information). The double dot was cycled through three configurations, depicted in Fig. 1e, while measuring the average QPC conductances. In the ‘empty’ (E) step, the second electron is removed, leaving a (0,1) state. In the ‘reset’ (R) step, a new second electron is added, initializing the (1,1) state to an unbiased mixture of the singlet, (1,1)S, and the three triplets (1,1) T_- , (1,1) T_0 , and (1,1) T_+ . In the ‘measurement’ (M) step, (0,2) is lowered relative to (1,1) until (0,2)S becomes the ground state, while the (0,2) triplets remain inaccessible, above the (1,1) states. Because tunnelling preserves spin, only (1,1)S can relax to (0,2)S, while the (1,1) triplets are spin-blockaded from making this transition^{25,26}.

The measurement step accounted for 80% of the pulse period (E and R were each 10%) so the time-averaged charge-sensor signal mainly reflects the charge state during the measurement time, t_M . Figure 1d shows g_{rs} as a function of the constant offsets to gate voltages V_L and V_R with pulses applied. The dashed lines indicate locations of ground-state transitions during the M step, as seen in unperturbed double dots²². Gate pulses alter this signal only within the ‘pulse triangle’ (outlined by solid white lines). Here g_{rs} is intermediate between the (0,2) and (1,1) plateaus, indicating that although (0,2) is the ground state, the system is often stuck in the

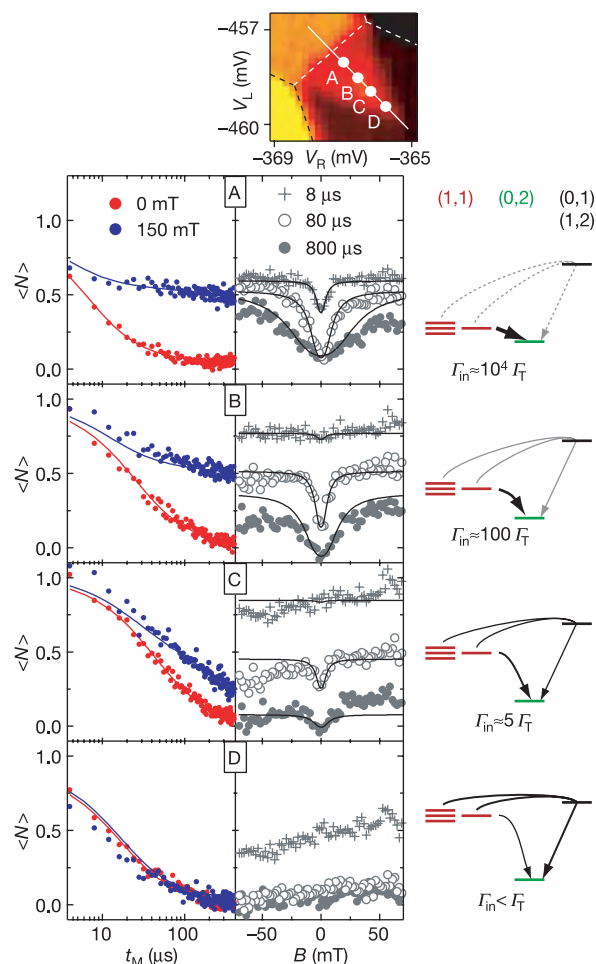


Figure 3 | Detailed measurements of blocked (1,1) occupation. Average occupancy $\langle N \rangle$ of the (1,1) charge state, based on calibrated charge sensor conductances, at four detuning points (labelled A, B, C, D in the uppermost panel). Left panels show $\langle N \rangle$ as a function of t_M at $B = 0$ and $B = 150$ mT. Middle panels show $\langle N \rangle$ as a function of B for different t_M times. Diagrams at right show schematically the relative position of energy levels and the extracted ratios of inelastic (Γ_{in}) to thermal (Γ_T) decay rates.

excited (1,1) state. In the regions labelled M' and M'' , alternate, spin-independent relaxation pathways, shown in Fig. 1f, circumvent the spin blockade.

The magnetic field, B , and t_M dependence of the charge sensor signal is shown in Fig. 2. With $t_M = 8 \mu s$, a large signal is seen in the pulse triangle, indicating that some of the (1,1) to (0,2) transitions are spin blocked. As t_M is increased this signal decreases (Fig. 2b), indicating that t_M is approaching the (1,1) singlet–triplet relaxation time. This is accompanied by a reduction in the pulse triangle size due to thermally activated processes as in Fig. 1f. Similar data, but at $B = 0$, are plotted in Figs 2c and d. The signal in the pulse triangle is noticeably weaker for the same t_M , particularly near the (1,1)-to-(0,2) charge transition, indicating enhanced spin relaxation.

Detailed measurements of residual (1,1) occupation as a function of detuning (the energy difference between the (1,1) and (0,2) states) are shown in Fig. 3. Conductances g_{ls} and g_{rs} were measured along the diagonal white line in the upper panel of Fig. 3, for various values of B and t_M , and converted to occupancy $\langle N \rangle$ by scaling to the average (1,1) and (0,2) levels outside the pulse triangle. Data are shown in detail for the points labelled A through D. As in Fig. 2, strong field dependence was found at low detuning (point A), where

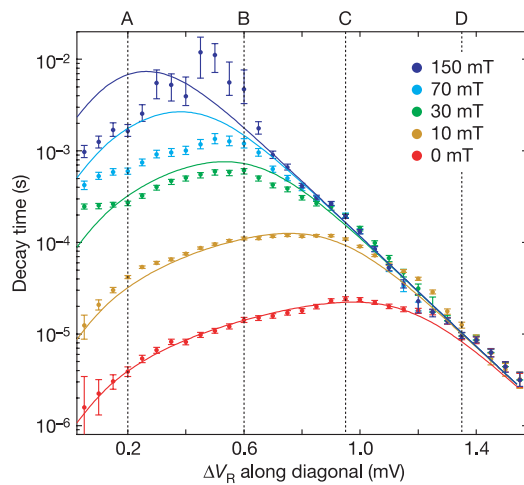


Figure 4 | Decay of (1,1) occupancy as a function of detuning at various magnetic fields. Dotted lines mark the locations of points A through D from Fig. 3. Fit of zero field theory (red curve) to data (red circles) sets all fit parameters except B_{nuc} , which is determined by fitting to the 10-mT data (gold). Theory curves at other fields are then fully determined. Error bars at zero field result from the least-squares fitting. Error bars at non-zero field reflect changes in the resulting decay rate as the zero-field fitting parameters are varied within their uncertainties.

inelastic interdot tunnelling dominates. This field dependence vanishes at higher detuning where thermally activated tunnelling to the leads dominates.

As in previous work^{4,11}, we model spin evolution in (1,1) by treating the ensemble of nuclear spins within each dot as a static effective field $\mathbf{B}_{\text{nuc}}$ with slow internal dynamics, that adds to any applied Zeeman field (see Fig. 1b). $\mathbf{B}_{\text{nuc}}$ is randomly oriented with root mean square strength $B_{\text{nuc}} = b_0 \sqrt{I_0(I_0 + 1)/N_{\text{nuc}}}$, where $b_0 = 3.5$ T is the hyperfine constant in GaAs, $I_0 = 3/2$ is the nuclear spin and N_{nuc} is the effective number of nuclei with which the electron interacts^{2,3,27,28}. In our dots, N_{nuc} is estimated at 10^6 – 10^7 , giving $B_{\text{nuc}} \approx 2$ – 6 mT. The spins precess in a characteristic time $t_{\text{nuc}} = \hbar/g^* \mu_B B_{\text{nuc}} \approx 3$ – 10 ns, which can be regarded as an inhomogeneous dephasing time T_2^* . At $B = 0$, all four (1,1) spin states mix in this time, and tunnelling will appear insensitive to spin. With $B > B_{\text{nuc}}$, however, only (1,1) T_0 and (1,1) S are degenerate. These will continue to mix with the same rate, but (1,1) T_+ and T_- will be frozen out.

To model this mixing, we assume static nuclear fields during each pulse, a spin-preserving inelastic interdot tunnelling rate Γ_{in} from (1,1) S to (0,2) S , and a spin-independent rate Γ_{T} due to thermally activated tunnelling via the (0,1) and (1,2) charge states (see Supplementary Information for details). Zeeman eigenstates for two spins in fields $B\hat{z} + \mathbf{B}_{\text{nuc},\text{L}}$ and $B\hat{z} + \mathbf{B}_{\text{nuc},\text{R}}$, denoted $|(1,1)s,s'\rangle$ ($s, s' = \pm 1/2$), decay to (0,2) S on the basis of their overlap with (1,1) S , with rates $\Gamma_{ss'} = \Gamma_{\text{in}} | \langle (1,1)S | (1,1)s,s' \rangle |^2$ as long as $\Gamma_{\text{in}} \ll g^* \mu_B B_{\text{nuc}}$. Averaging over nuclear field configuration and short-time dynamics gives decay rates for the $T_{\pm}$ -like states:

$$\Gamma_{\pm 1/2, \pm 1/2} = \frac{\Gamma_{\text{in}}}{4(1 + (B/B_{\text{nuc}})^2)} \quad (1)$$

and $\Gamma_{\pm 1/2, \mp 1/2} = \Gamma_{\text{in}}/2 - \Gamma_{\pm 1/2, \pm 1/2}$ for the S -like and T_0 -like states. At $B = 0$, total transition rates for all (1,1) states into (0,2) S are the same, $\tau_0^{-1} = \Gamma_{\text{in}}/4 + \Gamma_{\text{T}}$. For $B > B_{\text{nuc}}$, transition rates $\tau_B^{-1} = \Gamma_{\pm 1/2, \pm 1/2} + \Gamma_{\text{T}}$ from (1,1) $T_{\pm}$ to (0,2) S are suppressed by field, while transitions from (1,1) S and (1,1) T_0 to (0,2) S are accelerated by up to a factor of two because they no longer mix with (1,1) $T_{\pm}$. During the gate-pulse transition from R to M, the relatively fast transition from (1,1) S to (0,2) S allows a fraction q of the (1,1) S state to transfer

adiabatically to (0,2) S , reducing the initial occupation of (1,1) S . The resulting average occupancy N of (1,1) after a time t_M is:

$$N(t_M) = \frac{1}{t_M} \int_0^{t_M} dt \left(\frac{1}{2} e^{-t/\tau_B} + \frac{2-q}{4} e^{-t(2\tau_0^{-1} - \tau_B^{-1})} \right). \quad (2)$$

Experimentally measured values for N as functions of t_M and B for various detunings are shown in Fig. 3, along with fits to the above theory. An additional field-independent parameter, N_{∞} , accounts for non-zero $N(t_M)$ at long times owing to thermal occupation of (1,1). N_{∞} is zero at large detuning but increases, as expected, near zero detuning. Non-zero q values are found only at very low and very high detuning (where the R point is near zero detuning), where the slew rate of the pulse is low as it crosses to (0,2). With these parameters and τ_0 set for a given detuning by fitting the zero-field data (red), the high-field data (blue) are fitted with just the longer decay times τ_B for the (1,1) $T_{\pm}$ states. The field-dependence curves (black) are then fully determined by B_{nuc} , which is most accurately determined from data in Fig. 4, as discussed below. Drift in sensor conductance over long field sweeps is compensated by allowing a vertical shift in the field-dependence curves. The depth and width of the dips in these curves are not adjustable.

Figure 4 shows the extracted decay times τ_0 and τ_B versus detuning for various fields. As the magnetic field increases, more points at high detuning fall along a line in this semi-log plot, denoting exponential energy dependence as expected for a thermally activated process. This persists over three orders of magnitude at the highest field, and with calibration from transport measurements yields a temperature of 160 ± 20 mK. At zero field, thermal decay dominates only at the highest detunings, and the low-detuning times are well fitted by a power-law function of detuning with exponent 1.2 ± 0.2 and offset 700 ns, typical of inelastic tunnelling in double quantum dots²⁹. Adding these two processes gives the red curve in Fig. 4, in good agreement with the zero-field data. The 10-mT curve is fitted using these zero-field parameters, but with times for the inelastic component increased by the factor $(1 + (B/B_{\text{nuc}})^2)$ from equation (1). The fit gives $B_{\text{nuc}} = 2.8 \pm 0.2$ mT, or $N_{\text{nuc}} \approx 6 \times 10^6$, within expectations. This value uniquely determines the remaining theory curves. For τ_B longer than about 1 ms the decay is faster than theory predicts (though still 10^3 times slower than at $B = 0$), indicating that another mechanism such as spin-orbit coupling may operate on millisecond timescales^{8–10}. Spin-orbit coupling is expected to dominate spin relaxation at external fields of several tesla⁹. This regime is better suited to parallel fields, which couple almost exclusively to spin, than to the perpendicular orientation used here, which affects orbital wavefunctions at high fields.

Given B_{nuc} above, the model predicts an inhomogeneous dephasing time $T_2^* \approx 9$ ns for this device, which is independent of external field despite the enhanced relaxation times measured at higher fields. Up to 1 ms, the excellent agreement between experiment and theory indicates that hyperfine interaction is the only relevant source of spin relaxation in this system. Several strategies are available to circumvent this short dephasing time. Materials with zero nuclear spin, such as carbon nanotubes, avoid hyperfine effects entirely. Controlling $\mathbf{B}_{\text{nuc}}$ via nuclear polarization^{11,17} is tempting, but high polarization is required for T_2^* to increase substantially³⁰. An alternative is to use spin echo techniques such as pulsed electron spin resonance to extend coherence to the nuclear spin correlation time, expected to be of the order of 100 μ s in these devices⁴.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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The breakdown of continuum models for mechanical contacts

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Forces acting within the area of atomic contact between surfaces play a central role in friction and adhesion. Such forces are traditionally calculated using continuum contact mechanics¹, which is known to break down as the contact radius approaches atomic dimensions. Yet contact mechanics is being applied at ever smaller lengths, driven by interest in shrinking devices to nanometre scales^{2,3}, creating nanostructured materials with optimized mechanical properties^{3,4}, and understanding the molecular origins of macroscopic friction and adhesion^{5,6}. Here we use molecular simulations to test the limits of contact mechanics under ideal conditions. Our findings indicate that atomic discreteness within the bulk of the solids does not have a significant effect, but that the atomic-scale surface roughness that is always produced by discrete atoms leads to dramatic deviations from continuum theory. Contact areas and stresses may be changed by a factor of two, whereas friction and lateral contact stiffness change by an order of magnitude. These variations are likely to affect continuum predictions for many macroscopic rough surfaces, where studies^{7,8} show that the total contact area is broken up into many separate regions with very small mean radius.

One assumption of continuum mechanics is that the displacements of discrete atoms can be represented by continuously varying strain fields and related to the internal stress by the bulk elastic moduli¹. There is evidence that this approximation remains surprisingly accurate even at the scale of a few atomic diameters^{9–11}. Studies of contact also assume that surfaces are perfectly flat at sufficiently small scales, but the limits of this assumption are rarely considered. As shown below, the atomic structure of surfaces necessarily leads to bumps whose radii of curvature are comparable to an atomic diameter. Moreover, many experiments suggest that macroscopic surfaces become steeper and more curved at smaller length scales^{5,7,12}. The applicability of contact mechanics to such surfaces is unclear.

Experimental tests of continuum mechanics in nanometre-scale contacts are difficult. Scanning probe microscopes (SPM) measure total forces and displacements with tips of radius $R \approx 10\text{--}100\text{ nm}$, but other quantities, such as contact area, must be inferred from friction or conductivity measurements, or estimated from theory^{5,13–19}. One motivation of this paper is to quantify the type of errors that may result from such analysis.

We consider geometries that are easy to treat in both continuum theory and atomistic simulations: contact between a rigid cylinder or sphere of radius R and a flat elastic substrate. Within continuum theory this is equivalent to contact between two elastic surfaces¹. Tests of this equivalence and further details of the simulations are provided in the Supplementary Information.

The substrate is a face-centred cubic (f.c.c.) crystal with a (001) surface, reduced modulus E^* , and volume per atom σ^3 , where σ represents an effective atomic diameter. We present results for ideal harmonic crystals, but find similar results for Lennard–Jones interactions (Supplementary Information). Periodic boundary

conditions are applied along the surface of the substrate and the bottom is held fixed. For cylinders (Fig. 1) the results are averaged over the spatial period $l (\geq 10\sigma)$ along the axis. All other substrate dimensions are much larger ($\geq 200\sigma$), to limit boundary effects.

Three models for the atomic structure of surfaces are considered (Fig. 1). All are identical from the continuum perspective, deviating from a smooth arc by at most σ . The smoothest is a slab of f.c.c. crystal bent into a cylinder. We contrast results for cylinders made by bending crystals with the same atomic spacing as the substrate (commensurate) and an irrational multiple (incommensurate). Amorphous and stepped tips were obtained by cutting cylinders from a bulk glass or crystal. We focus on a radius typical of SPM tips, $R = 100\sigma$ ($\sim 30\text{ nm}$), but studied R up to $1,000\sigma$. Real tip geometries are probably much rougher, but seldom characterized¹³. Atoms on the cylinder and substrate interact with a Lennard–Jones force that, unless noted, is truncated to eliminate adhesion (Supplementary Information).

We first examine the normal tip displacement δ of cylindrical tips as a function of normal force N . Figure 2a shows that results for all surfaces are in quite good agreement with continuum theory¹, even though δ is less than 4σ . Except at very small loads, the main effect of atomic roughness is to uniformly increase δ . This shift represents the difference in height between the lowest atom and a typical atom, and is at most $\sim 0.25\sigma$. Experiments typically include the height at contact ($\delta = 0$) as a fit parameter¹⁷ and Fig. 2a implies that such fits yield accurate values for R and E^* ($\sim 10\%$).

Figure 2b shows that the errors in contact radius a are greater. Atomic roughness spreads the pressure over a wider region, and the

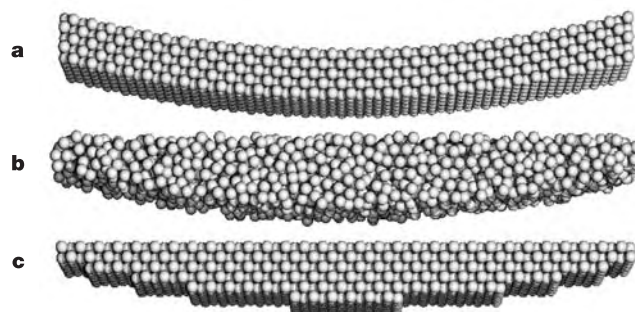


Figure 1 | Cylindrical surfaces with different atomic-scale roughness. Snapshots of atoms near the bottoms of cylindrical surfaces of average radius $R = 100\sigma$ formed by bending a crystalline slab (a), or cutting an amorphous (b) or crystalline solid (c). The steps in c are not a unique function of R , which leads to further variations in the behaviour of such tips. The tips are pressed down onto a horizontal elastic substrate. Periodic boundary conditions are applied along the axis of the cylinder, which runs into the page.

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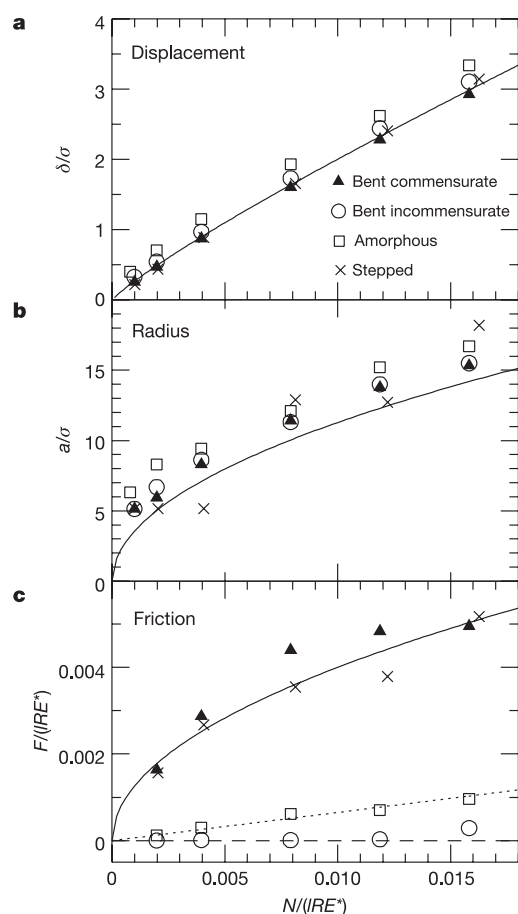


Figure 2 | Variation of normal displacement, contact radius and friction with normal load. Dimensionless plots of normal displacement δ (a), contact radius a (b) and static friction F (c) versus normal force N for the given cylinder surfaces (symbols). For stepped surfaces, a increases in discrete jumps. Here $R = 100\sigma$, the zero of δ corresponds to the first non-zero force, and a is half the range over which the force on substrate atoms is non-zero (Fig. 3). Solid lines show continuum predictions, with the assumption that F is proportional to area in c. The broken lines in c are linear fits.

resulting curves cannot be fitted accurately by any fixed values of R and E^* . A better description is provided by increasing the continuum prediction for a by a fixed amount (2 to 5σ) over the whole range of loads. The required increase in a rises with increasing R , doubling by $R = 1,000\sigma$. Similar plots are obtained for spherical tips (Supplementary Fig. S2). We conclude that continuum calculations may underestimate the area of SPM contacts by up to 100% at small loads, but that the fractional error decreases with increasing load and R .

Atomic-scale roughness may produce still larger deviations in local quantities. Figure 3 shows the variation of the pressure P along the contact. Results for bent incommensurate crystals lie closest to the solid lines predicted by contact mechanics, showing only a small smearing at the edge of the contact. Results for a bent commensurate crystal (Fig. 3b) show sharp drops in P where atoms on the substrate and cylinder fall out of registry. The pressure on the amorphous surface shows fluctuations that are comparable to the mean, even though the results are averaged along the cylinder axis. The local pressure for the stepped surface is farthest from the prediction, because its flat terraces differ most strongly from a smooth curve. Indeed, solving the contact mechanics problem for a flat contact gives a much better description of the pressure on a stepped surface, with a minimum in the centre and singularities at the edges¹. This,

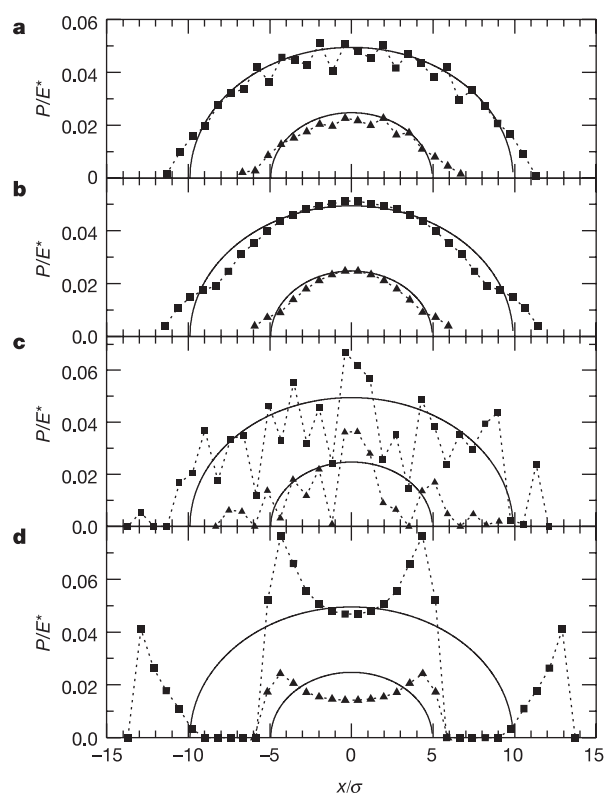


Figure 3 | Local pressure distributions for cylinders with different atomic-scale roughness. Pressure P is plotted as a function of lateral distance x from the cylinder axis for bent incommensurate crystal (a), bent commensurate crystal (b), amorphous cylinder (c), and stepped crystal (d). Results for two loads, $N/IRE^* = 0.0019$ (black triangles) and 0.0077 (black squares), are averaged along the length l of the cylinder axis and over bins of width σ along x . Solid lines show continuum predictions for the two loads, which are the same for all panels.

combined with previous work^{9–11}, suggests that continuum mechanics could be applied to smaller contacts if the true atomic-scale surface roughness was included, but such detail is seldom available and never used in practice¹³.

In continuum mechanics, Saint-Venant's principle implies that changing the surface distribution of normal load on the scale of a has little effect on stresses at depths below $3a$. Thus continuum predictions should grow in accuracy with increasing depth below the surface. However, the stresses are most significant at depths less than a . For example, continuum mechanics¹ predicts that the shear stress τ should peak $0.78a$ beneath the surface and directly below the cylinder axis. The local pressure deviations for amorphous and stepped surfaces in Fig. 3 lead to shifts in both the magnitude and location of the peaks in τ by factors of two or more. Values of the yield stress are often determined from the calculated subsurface value of τ at the onset of yield. We find that this may underestimate the yield stress by a factor of two or more because of the large local stresses below atomically rough surfaces.

Figure 4 and Supplementary Fig. S2 show that results for spherical tips with and without adhesion follow the same trends as non-adhesive cylinders. Bent crystal results with no adhesion fit well to Hertz theory. Adding adhesion produces a strongly peaked tensile ring whose location and magnitude fit the Maugis–Dugdale²⁰ model with independently determined values of E^* and work of adhesion w . Amorphous tips show strong fluctuations that nearly remove the tensile ring. The value of w is four times smaller than for a bent tip with the same interaction potential, and the contact radius is increased by several σ relative to continuum theory. Stepped

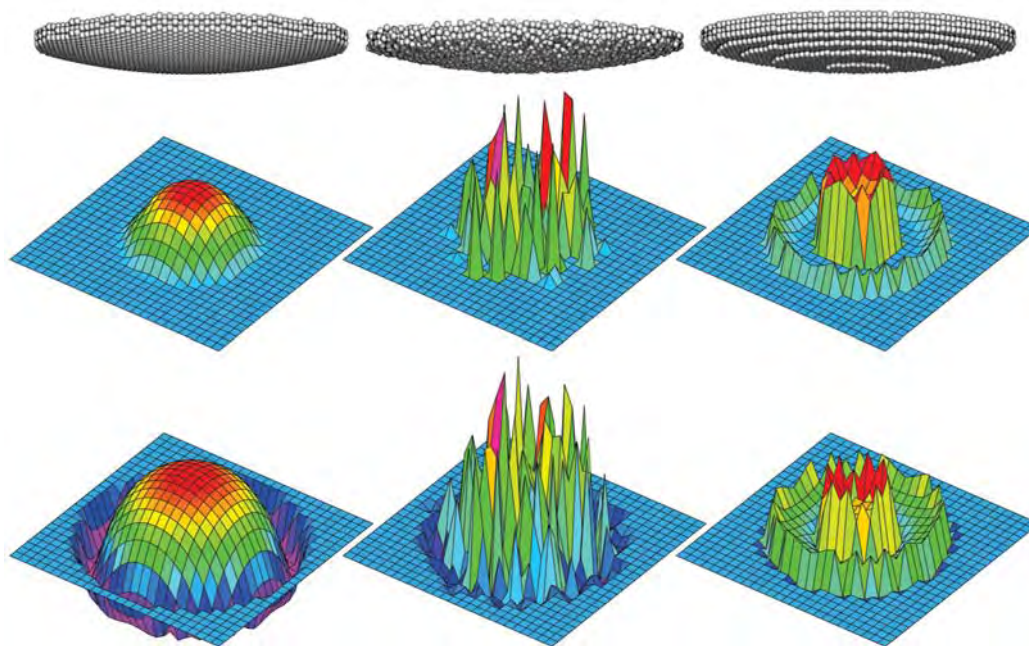


Figure 4 | Geometry and stress for spherical tips with and without adhesion. The top row shows the central regions (diameter 50σ) of bent, amorphous and stepped spherical tips, from left to right. The second and third rows show the pressure distributions for these tips with non-adhesive and adhesive interactions, respectively. In the adhesive case, the interaction

potential between surfaces is about half as strong as interactions within the substrate (Supplementary Information). In all cases $N/E^*R^2 = 0.0013$ and $R = 100\sigma$. The pressure is plotted over a central square region of edge 42.9σ , with a constant vertical scale. Almost 10^7 atoms are included in the substrate to include subsurface deformations.

tips show compressive peaks at step edges that look nothing like Maugis–Dugdale predictions.

Frictional forces can also be measured with the SPM and have been used to infer a relationship between area and friction^{13–16}. Analytic studies suggest that friction should be more sensitive to surface structure than other quantities²¹ and this is confirmed by our simulations. Figure 2c shows the static friction F needed to initiate sliding as a function of load. The bent and cut commensurate crystal exhibit large friction forces because they can lock into local registry with the substrate²¹. Results for both can be qualitatively fitted by assuming that friction is proportional to the area predicted by contact mechanics (solid line), even though the actual area is different. Similar fits have been made to some SPM experiments where the tip is unlikely to be commensurate^{13–16}.

The friction for amorphous and incommensurate surfaces is much smaller (Fig. 2c) and does not scale with the contact area because the atoms cannot lock into local registry at the surface²¹. The incommensurate surface exhibits almost no friction. This is consistent with SPM measurements with crystalline tips^{22,23}. The amorphous system appears to give friction proportional to load as in macroscopic friction laws^{1,21}. However, we found that F/N decreases as the cylinder length l increases, as predicted for bare amorphous surfaces^{21,24}.

The shear modulus of materials has also been determined with SPM by measuring the stiffness resisting lateral tip displacements^{13,18}. The results are analysed using continuum theory with the assumption that the surface atoms follow the tip rigidly. However, the small values of static friction for amorphous and incommensurate surfaces allow significant deviations between tip and substrate displacements. The effect is equivalent to adding another spring of stiffness of order F/σ in series with the continuum result¹⁹. This explains why the measured stiffness is often much less than expected¹⁸.

We note that contact mechanics has been tested for $R > 1$ mm and $a > 1$ μm where the contact area can be measured optically. However, these tests considered special cases that are consistent with our findings. Most surface force apparatus experiments^{25,26} used bent

mica crystals, the geometry that agrees best with continuum theory. As in Fig. 2b, a was actually slightly larger than continuum predictions, but this was attributed to systematic effects²⁵. Most other experiments have considered elastomers^{27–29}. While these materials are elastic at large scales, they can deform like fluids at lengths less than the cross-link spacing, allowing them to conform to atomic-scale roughness.

The above results have obvious implications for SPM experiments, suggesting that contact areas and yield stresses will be underestimated by continuum theory and that friction and contact stiffness will be overestimated. There is evidence that the same effects may apply for typical macroscopic surfaces. Statistical analyses of random rough surfaces^{5,7,8,12} indicate that the typical radius of curvature R of contacting surface bumps is comparable to the smallest wavelength of height fluctuations and may be of nanometre scale. Work on quantifying these effects for complex rough surfaces is under way, and initial results show that the area may be more than twice that predicted using continuum mechanics.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Extent, duration and speed of the 2004 Sumatra–Andaman earthquake imaged by the Hi-Net array

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The disastrous Sumatra–Andaman earthquake of 26 December 2004 was one of the largest ever recorded. The damage potential of such earthquakes depends on the extent and magnitude of fault slip. The first reliable moment magnitude estimate¹ of 9.0 was obtained several hours after the Sumatra–Andaman earthquake, but more recent, longer-period, normal-mode analyses have indicated that it had a moment magnitude of 9.3, about 2.5 times larger². Here we introduce a method for directly imaging earthquake rupture that uses the first-arriving compressional wave and is potentially able to produce detailed images within 30 min of rupture initiation. We used the Hi-Net seismic array in Japan as an antenna to map the progression of slip by monitoring the direction of high-frequency radiation. We find that the rupture spread over the entire 1,300-km-long aftershock zone by propagating northward at roughly 2.8 km s^{-1} for approximately 8 minutes. Comparisons with the aftershock areas of other great earthquakes indicate that the Sumatra–Andaman earthquake did indeed have a

moment magnitude of ~ 9.3 . Its rupture, in both duration and extent, is the longest ever recorded.

Although aftershocks and an extended P-wave train suggested a 1,200-km-long rupture³, conventional source modelling using long-period body-wave and surface-wave seismograms constrained the bulk of the slip to the southern portion of the aftershock zone (Fig. 1a). A different approach to mapping the slip exploits the high-frequency energy generated during rupture propagation. Hi-Net, a dense seismic array in Japan⁴, consists of about 700 short-period borehole instruments located throughout Japan at $\sim 20 \text{ km}$ spacing (Fig. 1b). The array spans distances from 43° to 60° and azimuths from 36° to 47° with respect to the Sumatra–Andaman mainshock epicentre. The P-wave onset of the event is remarkably coherent among different stations in the array (Fig. 1c), but later parts of the P wavetrain are complicated by multiple, overlapping arrivals of seismic energy from different portions of the rupture. Because changes in the source location cause changes in the relative arrival

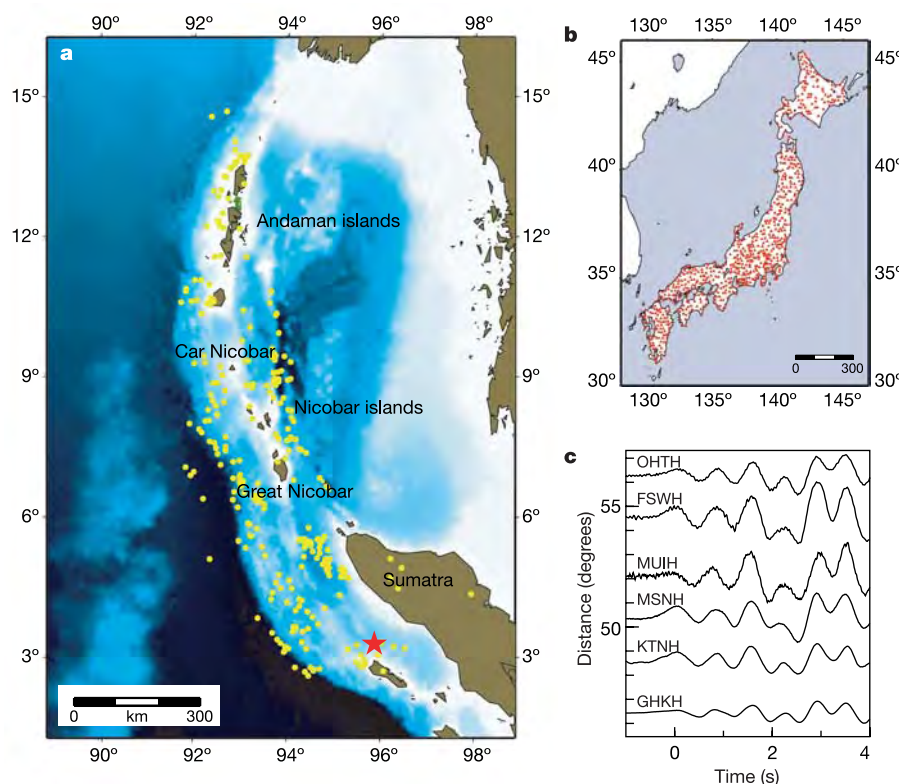


Figure 1 | Earthquake and station distribution. **a**, The region of the 26 December 2004 earthquake epicentre (red star) and aftershock locations (yellow dots). Seafloor bathymetry is shown as the background, with lighter colours for shallower regions. **b**, Distribution of ~ 700 stations (small red triangles) throughout Japan that comprise the Hi-Net seismic array. **c**, Examples of the initial (4 s) P-wave arrivals recorded on the vertical components at varying distances from the hypocentre. Station names are given with each trace.

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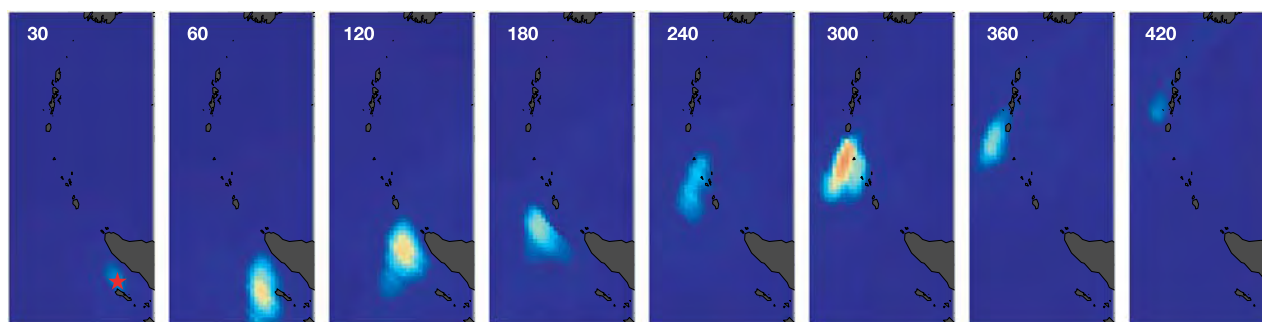


Figure 2 | Rupture progression. Maps showing the distribution of energy radiation at 30-s intervals for the first minute, and at 1-min intervals thereafter, following the earthquake initiation. Note the northward

migration of the rupture during the ~8-min-long event. The epicentre is indicated by the red star in the first panel. The spacing for the source grid used to stack seismograms is 0.2° in both latitude and longitude.

times across the array, these complications can be unravelled to image the distribution of the high-frequency radiation during rupture.

Our analysis applies a back-projection method in which seismograms are stacked for each possible source location to obtain a direct image of the source^{5,6} (see Methods). The stacking procedure sums the energy that is radiated from the given source point constructively, and cancels out other energy present in the seismograms. Resulting maps showing the squared amplitudes of the stacks, which are proportional to radiated seismic energy in the short-period band of the data (~1–5 s), are shown in Fig. 2 at progressive times, and the peak location and amplitude as a function of time are shown in Fig. 3. At time zero, the earthquake starts at the epicentre, just west of northern Sumatra. There is a major burst of radiated energy about 80 s later as the rupture progresses northwest (Fig. 3b). A second peak occurs at about 300 s, west of Car Nicobar where there was a

tsunami-generating earthquake on 31 December 1881 with an estimated magnitude of 7.9 and a recurrence time of 114–200 yr (ref. 7). In the first 200 s, our analysis of high-frequency array data agrees with preliminary spatio-temporal slip-distribution models based on long-period global data. However, our rupture model lasts longer, for about 8 min (Fig. 3b), and extends farther to the north, into the Nicobar and Andaman islands region. The slip is unilateral with a sub-shear-wave speed of $\sim 2.8 \text{ km s}^{-1}$ (Fig. 3a).

To illustrate the total slip area, the distribution of cumulative

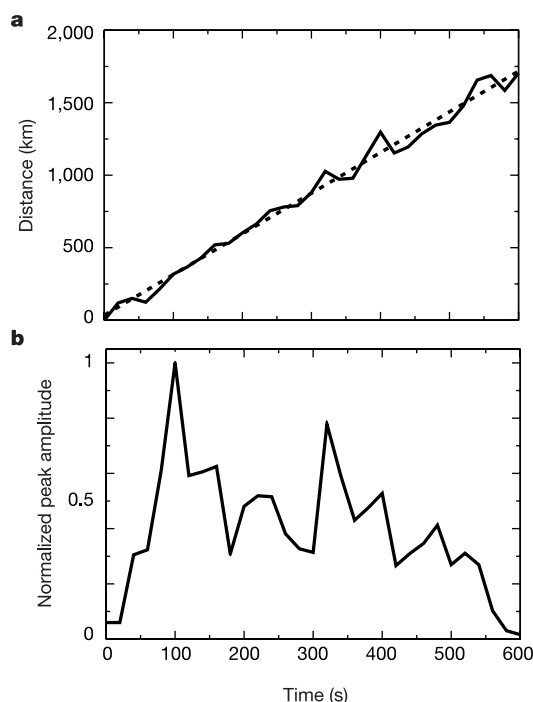


Figure 3 | Rupture speed and energy release. **a**, Rupture distance along the fault versus time. The dashed line is the straight-line fit to the peak locations, and gives an average rupture speed of 2.8 km s^{-1} . Distances beyond 1,300 km are not reliable because of the diminishing peak amplitudes beyond 500 s. **b**, Normalized peak amplitude as a function of time, showing two significant high-frequency energy events at ~80 s and 300 s.

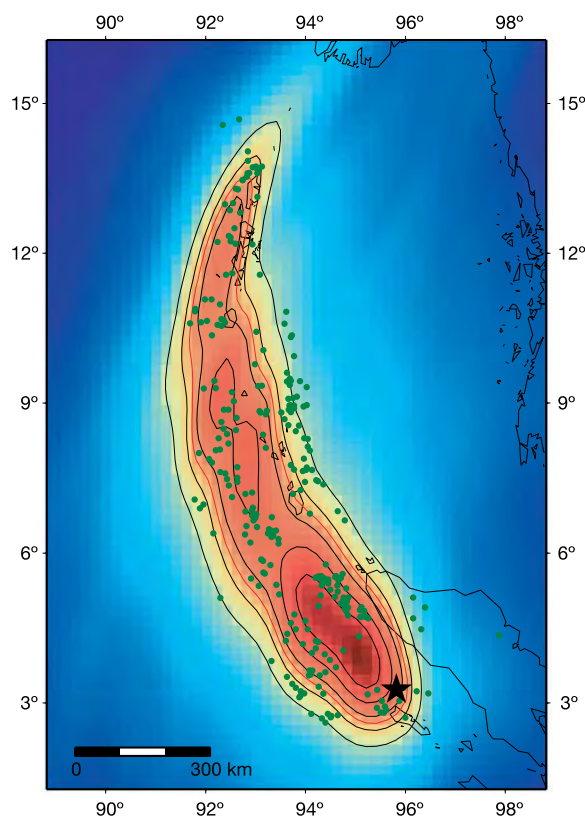


Figure 4 | Cumulative radiated energy. Integrated seismic energy over 600 s after initiation, normalized such that the maximum value is unity. The red contour, plotted at 65% of the maximum, encloses the slip area used to estimate the moment magnitude. The epicentre is shown as the black star. Note the good agreement between the 1,300-km-long rupture zone and the locations of the first month of aftershocks (dark green circles). The black contours are plotted at increments of 0.1 starting at 0.5. The image is computed and shown across the entire map, but amplitudes are very weak outside the contoured region.

radiated energy in the 600 s from the start of the earthquake is shown in Fig. 4. The slip is strongest in the southern portion west of northern Sumatra, but there is also significant radiation in the northern portion west of the Nicobar and Andaman islands. Because time shifts due to three-dimensional structure are derived from the initial waveforms associated with the hypocentre, the amplitudes towards the north end of the rupture are underestimated to some extent by incoherent stacking, and the northern peak may be as high as or higher than the southern peak. It is interesting to note that the largest aftershock (magnitude 7.1), 3.5 h after the mainshock, occurred west of Great Nicobar island where there is a local amplitude minimum in our model.

One test for the reliability of a rupture model is to compare its area to the aftershock distribution, because aftershocks generally occur near the mainshock slip surface. In Fig. 4, aftershock locations are shown by dark green circles, and their locations agree well with our image of the source of the high-frequency radiated energy. The 2004 Sumatra–Andaman earthquake has a comparable aftershock area to the three other largest earthquakes that have been adequately recorded; the 1957 Aleutian earthquake, the 1960 Chile earthquake, and the 1964 Alaskan earthquake (Fig. 5). The aftershock zones have markedly different aspect ratios owing to variations in rupture width and dip of the fault plane. Figure 5 demonstrates that the 2004 earthquake has the longest extent, about 1,300 km, consistent with our imaging. Similarly, the duration of rupture (about 500 s) is significantly longer than that of any known earthquake; average durations from short-period records for the Chile (13 stations) and Alaska (19 stations) events were ~ 345 s and ~ 340 s, respectively⁸. Estimates of rupture speed for the older earthquakes are somewhat uncertain but consistent; Chile broke dominantly unilaterally at ~ 3.5 km s⁻¹ (ref. 9), Alaska broke unilaterally at ~ 3.0 km s⁻¹ (ref. 10), and the Aleutian was largely unilateral with poorly constrained rupture speed¹¹. Farther west along the Aleutian arc, the 1965 Rat islands earthquake with moment magnitude ~ 8.7 ruptured at 2.7–2.9 km s⁻¹ (ref. 12). Unilateral rupture is a general tendency of large earthquakes¹³, and the sub-shear-wave speeds of these earthquakes are roughly consistent with predictions for mode III crack propagation¹⁴.

Slip area can provide a quick but crude estimate of seismic moment. We estimate from our imaging results that the slip area

of the Sumatra–Andaman event was about 210,000 km² (area enclosed by the red contour on Fig. 4). The area defined by the first month of aftershocks is about 360,000 km². To correct for the expansion of the aftershock zone following a mainshock, this is reduced by a factor of 1.75 (ref. 15), resulting in a 1-day aftershock area of about 206,000 km², in agreement with our imaging result. Using an empirical relation¹⁵, these areas yield a moment of $\sim 1.3 \times 10^{23}$ N m and moment magnitude ~ 9.3 , in accord with recent normal-mode analysis². Thus, in slip area, the Sumatra earthquake is second only to the 1960 Chile event among great earthquakes in the last 100 yr, whereas in length and duration, it exceeds all historical events. However, the value for the moment will ultimately require reconciliation of long-period seismic constraints on the fault slip with the high-frequency rupture surface, geodetic measurements and tsunami modelling.

Our model provides the most detailed view yet of rupture propagation in a great earthquake, aided by the long fault plane and the favourable location of the Hi-Net array. The large-scale features of the earthquake appear simple: a unilateral slip with an average speed of about 2.8 km s⁻¹ and no secondary rupture. The long extent of the slip is also consistent with geodetic observations of island uplift and subsidence, which require substantial fault slip to the north (<http://cires.colorado.edu/~bilham/IndonesiAndaman2004.htm>). Because high-frequency radiation is likely to be accompanied by significant fault slip and moment release⁸, we find no evidence to support the slow slip model hypothesized for the northern part of the rupture².

One of the advantages of this array approach is that it requires no prior knowledge of fault geometry, fault dimension, or rupture duration. In addition, this observation-driven method takes advantage of the entire P wavetrain, and calculation of synthetic seismograms is not needed. It is insensitive to interference from later seismic phases such as PP, because their angle of incidence across the array is different from that of direct P, and short-period PP amplitudes are also significantly less than direct P, owing to strong upper-mantle attenuation^{3,16}. Finally, our approach provides more-detailed images of rupture timing and extent than are given by simple measures of short-period P-wave duration versus azimuth³. The success of our method for imaging the Sumatra–Andaman earthquake may, however, depend on the large dimensions of the earthquake and the large

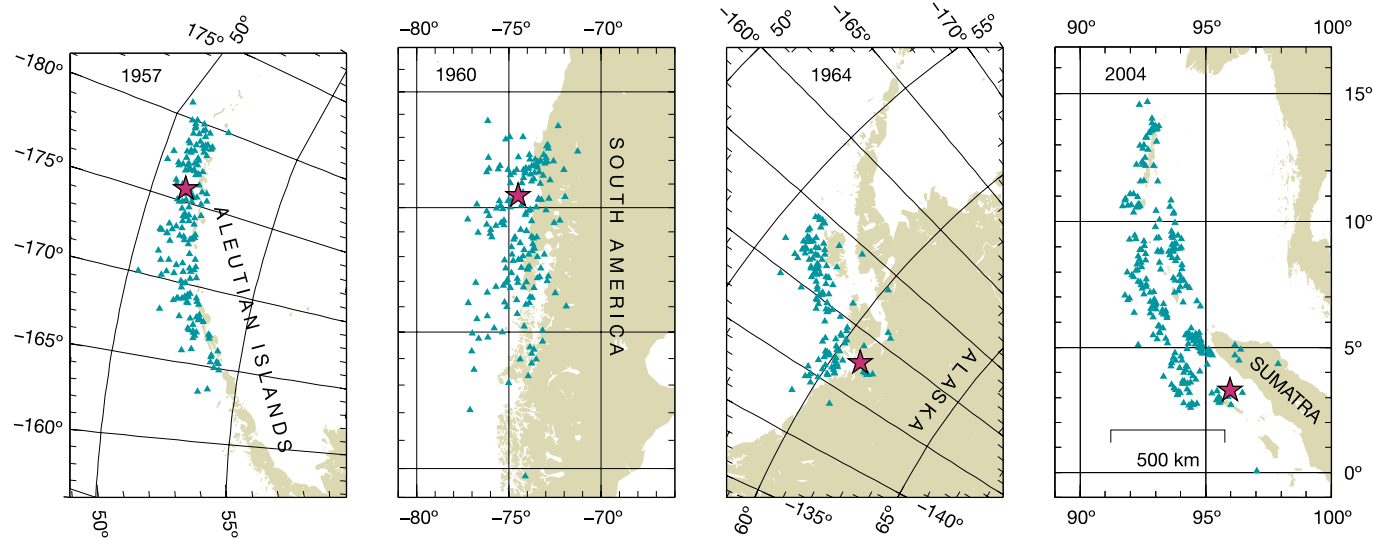


Figure 5 | Comparison of aftershock zones of great earthquakes. The epicentre of the mainshock is given by a red star, and aftershocks from the first month following the event are shown by blue-green triangles. All maps are at the same scale. The 2004 Sumatra–Andaman event has the longest aftershock zone, and an implied slip region that exceeds that of the 1957

$M_w \approx 9.0$ Aleutian¹⁸ and 1964 $M_w \approx 9.2$ Alaska¹⁹ events, and approaches that of the 1960 $M_w \approx 9.5$ Chile earthquake²⁰. For 1964 and 2004, all aftershocks of $M_w \geq 5$ are included; all available aftershocks are included for 1957 and 1960 as most events have no magnitude estimates.

number of high-quality records that were available for our study; its usefulness for smaller earthquakes or sparser networks is not yet clear.

Our results demonstrate the value of dense, high-quality seismic arrays, such as the Japanese Hi-Net, for monitoring global as well as local seismicity. Because our method applies to the first arriving P waves, it could be implemented in a real-time system in which a good estimate of the length and duration of great earthquakes could be obtained within 20 to 30 minutes of the earthquake origin time, depending on the distance of the array from the event. Such a system could be achieved at relatively modest cost, and would be a valuable component of worldwide tsunami warning programmes.

METHODS

Our back-projection method is a simplification of wavefield reverse-time migration, a tool for imaging structure in reflection seismology. For the j th source location, the seismograms are summed to make the stack s_j as a function of time t :

$$s_j(t) = \sum_k (p_k/A_k) u_k(t - t_{jk}^p + \Delta t_k)$$

where $u_k(t)$ is the vertical-component seismogram recorded at the k th station, and t_{jk}^p is the theoretical P-wave travel time from the j th source to the k th station¹⁷. Δt_k denotes timing corrections obtained from waveform cross-correlation of the initial 4 s of the P waves, which are used to enhance the coherence of the traces by accounting for effects due to three-dimensional structure. Finally, p_k and A_k are the polarity and amplitude of the seismograms obtained through cross-correlation analysis; the division by A_k ensures that the traces have approximately equal weight. The stacking procedure sums the energy that is radiated from the given source point constructively, and cancels out other energy present in the seismograms.

The records are de-measured, but no other filter is applied. To ensure waveform similarity, only seismograms with a correlation coefficient for the first 4 s of the P wave of greater than 0.7 with respect to a simple waveform stack are included in the analysis. This cut-off gives 538 seismograms out of 686 available traces. The stacking is performed over an evenly spaced grid of source latitude and longitude at 0.2° intervals, assuming a constant depth of 30 km. Differences in expected amplitudes from geometrical spreading, source depth variations and directivity effects are ignored, but they should be relatively minor. Estimates of the width of the rupture and the slip duration at fixed points are limited by the array geometry and the frequency content of the data (see Supplementary Information). The spatial resolution depends on the location, but the uncertainty in the rupture area generally takes the form of a 60 km by 170 km ellipse. The uncertainty in the rupture duration at fixed points along the fault is about 20 s.

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Tracking the rupture of the $M_w = 9.3$ Sumatra earthquake over 1,150 km at teleseismic distance

Frank Krüger^{1*} & Matthias Ohrnberger^{1*}

On 26 December 2004, a moment magnitude $M_w = 9.3$ earthquake occurred along Northern Sumatra, the Nicobar and Andaman islands, resulting in a devastating tsunami in the Indian Ocean region¹. The rapid and accurate estimation of the rupture length and direction of such tsunami-generating earthquakes is crucial for constraining both tsunami wave-height models as well as the seismic moment of the events. Compressional seismic waves generated at the hypocentre of the Sumatra earthquake arrived after about 12 min at the broadband seismic stations of the German Regional Seismic Network (GRSN)^{2,3}, located approximately 9,000 km from the event. Here we present a modification of a standard array-seismological approach and show that it is possible to track the propagating rupture front of the Sumatra earthquake over a total rupture length of 1,150 km. We estimate the average rupture speed to be $2.3\text{--}2.7\text{ km s}^{-1}$ and the total duration of rupture to be at least 430 s, and probably between 480 and 500 s.

For very large earthquakes, the observed seismograms at teleseismic distances contain mixtures of direct and later phases. Because most of the latter (such as PP) are of lower frequency content owing to their longer propagation path within the upper mantle, the direct phases can successfully be isolated by highpass filtering⁴. In this study we use directional properties of the wavefield characteristics, namely slowness and azimuth, which can be estimated with seismic arrays⁵. In addition to allowing for phase separation (see Supplementary Fig. 1), it permits the backprojection of the P-wave energy in space and time. This effectively allows us to localize the region of rapid slip for specific time windows.

Small-aperture seismic arrays situated in the vicinity of active faults were successfully used to track the propagating rupture front during large earthquakes^{6–8}. The spatial resolution of a seismic array is determined by its aperture and the bandwidth of the analysed signal⁵. Therefore, in a global monitoring context, the spatial resolution necessary for spatiotemporal imaging of source processes in the teleseismic distance range can only be achieved with large-aperture broadband arrays such as the 500 km by 700 km large GRSN². As standard array techniques based on the plane-wave assumption are of limited value for large aperture arrays³, we implemented a generalized curved wavefront stacking scheme to analyse the rupture characteristics of the Sumatra earthquake (also known as the Sumatra–Andaman islands earthquake).

For this purpose, the source region (0° to 20° latitude, 85° to 105° longitude) was spatially gridded into intervals of 0.2° along latitude and longitude at a fixed depth of 25 km (corresponding to the hypocentral depth). For each grid point the travel times of direct P waves to each station were calculated in a standard global Earth model⁹. One-minute-long segments of the unfiltered seismic traces were stacked along travel-time trajectories with respect to one reference station in the centre of the seismic array (MOX, Fig. 1).

The window length was chosen according to the dominant period of the P waves (20–30 s). Subsequently, the summation trace was squared and integrated to obtain the energy contribution from each grid point as proxy for the potential source strength. The temporal progression of the rupture was tracked by successively shifting the analysis window at the reference station in steps of 5 s for a total duration of 11 min. The origin time—at which an energy maximum was generated at the source—is calculated by subtracting the corresponding P-wave travel time from the time window centroid at the reference station. We note that owing to the progressive change in source position, the time separation between consecutive energy maxima varies, thus accounting for the directivity effect (reversing the directivity effect).

Figure 2a shows the resulting energy map for the time window

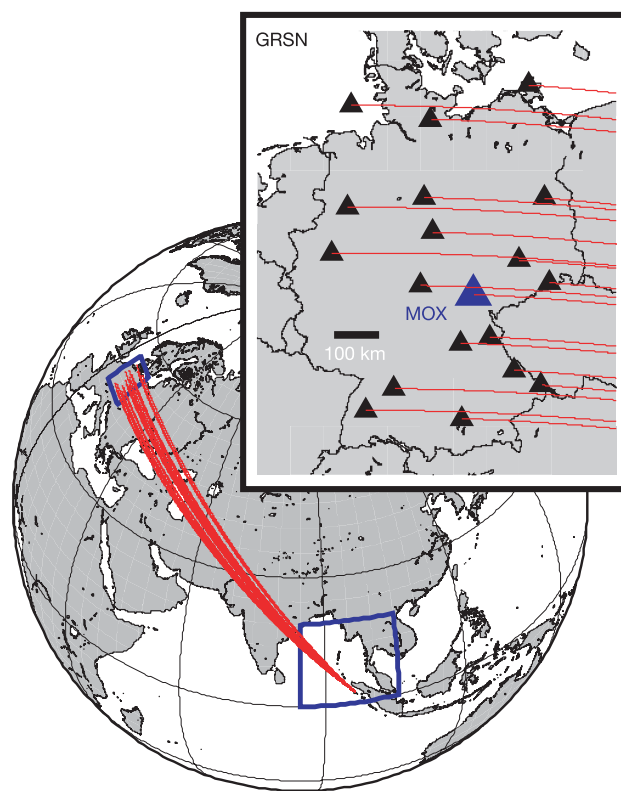


Figure 1 | Station distribution of the German Regional Seismic Network. Also shown are the corresponding great circle travel paths from the epicentre location of the Sumatra earthquake.

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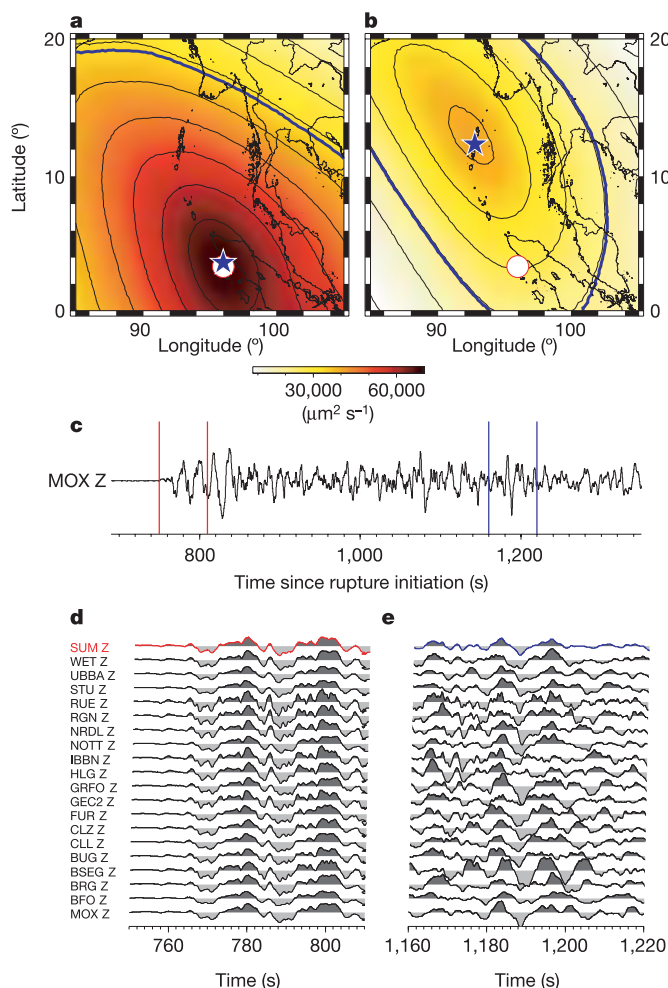


Figure 2 | Energy maps corresponding to initiation and end of the rupture during the Sumatra earthquake. **a**, Energy map for a time window enclosing the first P-wave arrival. **b**, Same as **a**, but for a time window 410 s later. White circle, NEIC epicentre; blue star, location of energy maximum. Blue 50% contour line depicts the array's capability to separate two seismic sources of similar strength acting at the same time. **c**, Seismogram recorded at the reference station. Red and blue bars mark time windows used to compute energy maps in **a** and **b**. **d**, **e**, Shifted seismogram segments and summation trace for **a** and **b**.

when the first P waves arrive at the network. The energy maximum (blue star) is located almost at the position of the epicentre (NEIC¹⁰ coordinates marked by a white circle). Figure 2b shows accordingly the energy map for a time window 410 s after the arrival of the first P wave at MOX. This corresponds to a time of 480 s after rupture initiation in the source region. The maximum of seismic energy generation has moved approximately 1,000 km northwards to the Andaman islands, showing that there was ongoing radiation of direct P-wave energy from the northern fault segment.

Figure 3 depicts the maxima of seismic energy obtained for each time frame. According to our analysis rupture initiated at latitude 3.4° N, longitude 95.8° E, which is consistent with the epicentral coordinates determined by NEIC¹⁰: latitude 3.3° N, longitude 96.0° E. During the first 60 s, the position of the energy maximum did not move. This possibly indicates a phase of bilateral growth of the rupture surface but cannot further be resolved owing to the limited spatial resolution of the array (compare the width of energy distribution indicated by the 50% contour line in Fig. 2a and b). Subsequently, the rupture travelled unilaterally along the trench to the north-northwest for about 600 km. Then, the rupture front

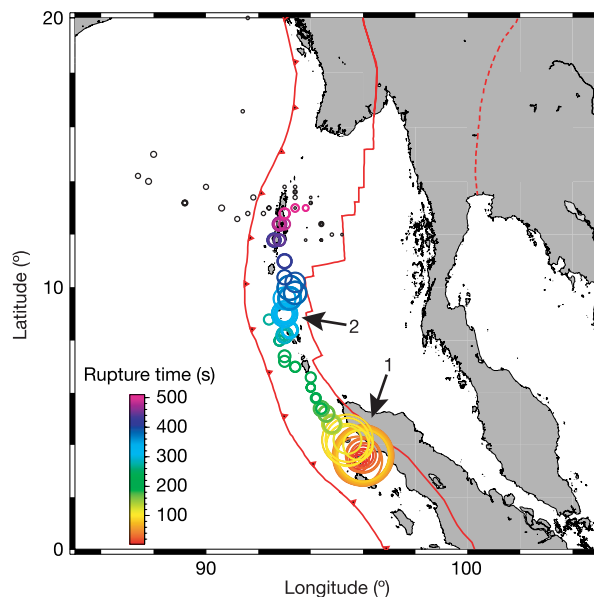


Figure 3 | Rupture trace of the Sumatra earthquake. Maxima of energy maps are shown in their spatiotemporal development by coloured circles. The width of the circles scales linearly with seismic energy; colour coding is proportional to the time since rupture initiation in the source region. Major tectonic lines are shown in red. Numbers 1 and 2 indicate the position of two major seismic energy releases.

changed its direction towards the north and continued to propagate for another 550 km till motion died out near the northern end of the Andaman islands.

After 300 s the first strong maximum of energy release is followed by a second energy release about one-third in amplitude, centred at latitude 9° N, longitude 93° E. It should be noted that the relative amplitude of these two energy-release peaks might be influenced by changes of the radiation pattern. However, the second energy release gives evidence that major rapid seismic slip occurred on the northern fault segment, consistent with the findings of refs 4 and 11. Our result also supports the conclusion of Stein and Okal¹² that the accumulated strain on the northern part of the rupture has been released during the Sumatra earthquake.

The total duration of the rupture was at least 430 s (end of the second strong energy release) and is probably as long as 480 to 500 s. Black circles in Fig. 3 mark energy sources located after 500 s. Their maximum energy is less than one-fifth of the second seismic energy-release peak amplitude. Similar results were obtained in refs 4 and 13.

The average rupture speed is estimated to be between 2.3 and 2.7 km s⁻¹. Uncertainty in rupture velocity estimates is mainly caused by the impracticality of uniquely specifying a time for the dominant contributing phase energy within the 60-s window length. The results suggest a slightly higher rupture velocity on the southern segment (2.4–2.8 km s⁻¹) when compared to the northern branch of the rupture (2.1–2.4 km s⁻¹). We note that the results for the southern branch may be additionally biased by the postulated (yet not resolvable) bilateral start of the rupture. That the rupture did not progress farther to the south-southeast despite high rapid slip at the beginning of the rupture may indicate that the rupture front hit a barrier in this direction. It may have been this barrier that broke three months later during the $M_w = 8.5$ earthquake on 28 March 2005.

METHODS

It is worth noting that the data of the used dense broadband seismic array in a distance of around 9,000 km proved optimal for the analysis, because the long direct P-wave train is not contaminated by very strong later phases such as the direct shear wave and its multiples. Spatial resolution can be increased by using more adjacent stations. Processing time for not optimized code on a standard PC

hardware was 1.5 times the data length. Considering the P-wave travel time, the data transmission time and the source duration, a complete estimate of the source extension would be available within about 30 min. The proposed technique could therefore aid decision-making within the first half-hour after very large seismic events.

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LETTERS

Cortical growth marks reveal extended juvenile development in New Zealand moa

Samuel T. Turvey^{1,2}, Owen R. Green³ & Richard N. Holdaway^{1,4}

Cyclical growth marks in cortical bone, deposited before attainment of adult body size, reflect osteogenetic changes caused by annual rhythms and are a general phenomenon in non-avian ectothermic and endothermic tetrapods¹. However, the growth periods of ornithurines (the theropod group including all modern birds) are usually apomorphically shortened to less than a year^{2,3}, so annual growth marks are almost unknown in this group⁴⁻⁶. Here we show that cortical growth marks are frequent in long bones of New Zealand's moa (Aves: Dinornithiformes), a recently extinct ratite order. Moa showed the exaggerated *K*-selected life-history strategy formerly common in the New Zealand avifauna, and in some instances took almost a decade to attain skeletal maturity. This indicates that reproductive maturity in moa was extremely delayed relative to all extant birds. The two presently recognized moa families (Dinornithidae and Emeidae) also showed different postnatal growth rates, which were associated with their relative differences in body size. Both species of giant *Dinornis* moa attained their massive stature (up to 240 kg live mass) by accelerating their juvenile growth rate compared to the smaller emeid moa species, rather than by extending the skeletal growth period.

Cortical bone deposition can be temporarily slowed or halted in response to seasonal periodicity, resulting in bone compacts being interrupted by dense annuli and/or lines of arrested growth (LAGs), which show progressively closer spacing towards the periosteum¹. Such growth marks are known in Mesozoic birds⁴, but have been reported in ornithurines only from long bones of the extant parrot *Amazona*, the recently extinct giant moa *Dinornis*, and the Eocene gruiform *Diatryma*^{5,6}. The presence of growth marks in these taxa has been used to challenge the idea that LAGs indicate low rates of metabolism or growth, and suggest instead that they might be retained plesiomorphic features^{2,5}. Moreover, cortical growth lines are absent even in polar bird species that experience extreme temporal interruptions to their skeletal development⁷, and have so far proved impossible to induce experimentally in birds⁶. Other studies have failed to detect such structures in moa^{8,9}. Growth marks in ornithurines are widely known only in the distinct endosteal and periosteal 'late bone' tissues deposited during adult bone growth, the skeletochronological usefulness of which remains controversial^{10,11}. They have therefore been of little use so far in studying developmental aspects of ornithurine autecology, such as postnatal ontogeny or life-history strategy.

Table 1 | Specimens and growth mark data

Species	Specimen number*	Element†	Locality‡	Annuli/LAGs
Dinornithidae				
<i>Dinornis novaezealandiae</i>	MNZ S 145	TT	Makirikiri (N)	0
	MNZ S 24365	TT	Takapau Road (N)	3
<i>D. robustus</i>	MNZ S 38988	TT	Takaka (S)	0
	CM AV 13779	F	Pyramid Valley (S)	0§
	MNZ S 39964	TM	Bell Hill Vineyard Swamp (S)	0
Emeidae				
<i>Anomalopteryx didiformis</i>	CM AV 19187	TT	Metro Cave, Punakaiki (S)	4
	MNZ S 202	TT	Broken River (S)	8
<i>Emeus crassus</i>	CM AV 8317	F	Pyramid Valley (S)	0
<i>Euryapteryx curtus</i>	KM 2003/1	F	Tokerau Beach (N)	4
	KM 2003/2	F	Tokerau Beach (N)	3
<i>Eu. geranoides</i>	CM SB282	F	Cheviot (S)	9
<i>Megalapteryx didinus</i>	CM AV 9089	TT	Glenmark (S)	3
	CM AV 10348	TT	Takahe Valley (S)	4
	CM AV 23589	F	Tautuku Beach (S)	5
	CM AV 31596	F	Annandale (S)	5
	MNZ S 445	F	Takahe Valley (S)	0
	W 0103	TT	Mt Luxmore (S)	0§
	CM AV 39341	TM	Mt Arthur (S)	1§
<i>Pachyornis elephantopus</i>	CM AV 38563	TM	Gowan Hills (S)	0
	MNZ S 40023	TT	Bell Hill Vineyard Swamp (S)	0
<i>P. mappini</i>	AM 6020	F	Waikaremoana (N)	0

* AM, Auckland Museum; CM, Canterbury Museum; KM, Kaitia Museum (for KM, specimen numbers from samples in Henry Wellcome Ancient Biomolecules Centre, University of Oxford); MNZ, National Museum of New Zealand Te Papa Tongarewa; W, Waitomo Museum.

† F, femur; TT, tibiotarsus; TM, tarsometatarsus.

‡ N, North Island; S, South Island.

§ Specimen shows extensive secondary remodelling.

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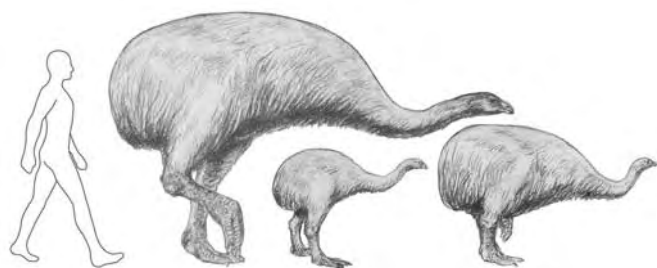


Figure 1 | Reconstructions of dinornithid and emeid moa species. A 1.8-m tall person is used as a scale. Left to right: female *Dinornis novaeseelandiae* (Dinornithidae), *Megalapteryx didinus* (Emeidae), *Pachyornis elephantopus* (Emeidae). Images courtesy of Colin Edgerley and New Zealand Geographic.

Moa represent a remarkable adaptive radiation of ratites that occupied a variety of large-herbivore niches in New Zealand in the absence of native mammalian competitors (Fig. 1). They all became

extinct soon after the arrival of Polynesian colonists about 700 years ago¹². Extreme female-biased sexual size dimorphism, originally suggested in the nineteenth century¹³, has recently been confirmed in many moa species by studies of ancient DNA^{14,15}. Females of the two *Dinornis* allospecies (*D. novaeseelandiae* and *D. robustus*) were among the largest known birds, measuring about 2 m tall at the back and weighing up to 240 kg, whereas males weighed only 34–85 kg (ref. 16). During the Holocene, the largest emeid moa, *Pachyornis elephantopus*, averaged 80 kg, and most other emeid species were substantially smaller¹⁶. In comparison, ostriches (*Struthio camelus*), the largest living ratites, weigh 90–130 kg (ref. 3).

To investigate the nature, frequency and phylogenetic distribution of cortical growth marks within the Dinornithiformes, we prepared a total of 21 histological thin sections from the midshaft region of long bones (femur, tibiotarsus, tarsometatarsus) of individuals that had attained adult body size, for nine out of the ten currently recognized moa species (Table 1). All specimens, except for a tarsometatarsus of *Dinornis robustus* (MNZ S 39964), have a non-fibrous bone surface texture marked by blood vessel impressions and tuberosities, representing the adult 'late growth' periosteal layer¹⁷ and indicating that

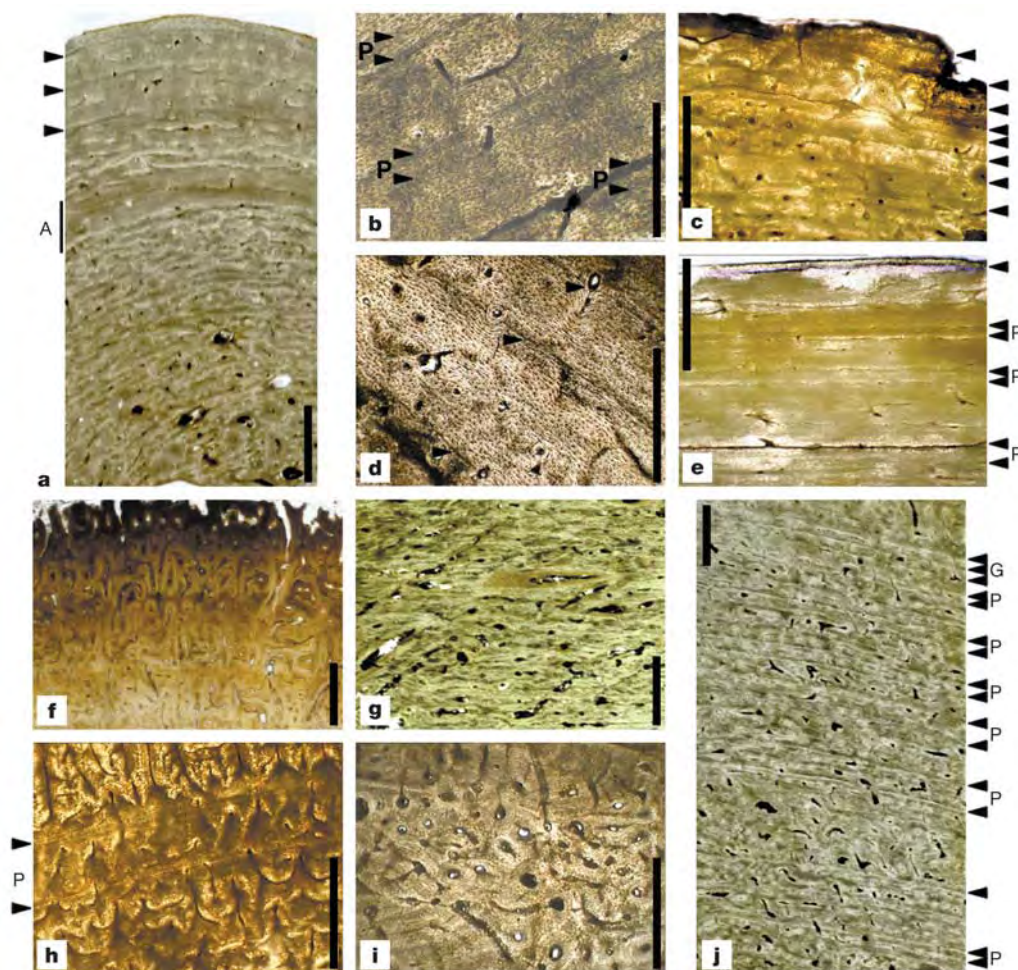


Figure 2 | Transverse undemineralized diaphyseal thin sections through moa long bones. The bone periphery is at the top. All specimens are from adult individuals except where indicated. **a**, *Megalapteryx didinus* tibiotarsus (CM AV 10348). **a**, Cortical cross-section, showing a lamellar annulus (A) in the mid-cortex and three LAGs (arrows) in a second annulus in the outer cortex. **b**, Close-up view of three paired LAGs. **c**, *Anomalopteryx didiformis* tibiotarsus (MNZ S 202), showing eight single LAGs. **d**, *M. didinus* tibiotarsus (CM AV 9089), showing three single LAGs. **e**, *A. didiformis* tibiotarsus (CM AV 19187), showing four single or paired LAGs. **f**, Sub-adult *Dinornis robustus* tarsometatarsus (MNZ S 39964),

showing fast-growing fibrolamellar matrix. The transverse coloured bands represent tidemark stains caused by repeated penetration of swamp fluid, not growth marks. **g**, *Pachyornis elephantopus* tarsometatarsus (CM AV 38563), showing poorly vascularised lamellar-zonal matrix lacking annuli or LAGs. **h**, *D. novaeseelandiae* tibiotarsus (MNZ S 24365), showing a pair of annuli each containing a LAG. **i**, *D. novaeseelandiae* tibiotarsus (MNZ S 145), showing highly vascularised fibrolamellar matrix lacking annuli or LAGs. **j**, *Euryapteryx geranoides* femur (CM SB282), cortical cross-section showing eight single, paired (P) or grouped (G) LAGs. Scale bar, 1 mm (**a**, **c**, **e**–**j**), 0.5 mm (**b**, **d**).

they are comparable mature growth stages. The bones of both *Dinornis* allospecies show highly vascularized primary fibrolamellar bone compacta, with a woven bone matrix containing reticular or longitudinal vascular canals (Fig. 2f, h, i). This structure matches the condition shown by living ratites^{4,17} and other birds⁵. Emeid compacta are instead characterized by extensive regions of primary zonal bone tissue, consisting of interrupted parallel-fibred and/or lamellar tissue containing few, simple, longitudinal vascular canals, which becomes increasingly avascular towards the periosteum and dominates the outer cortex (Fig. 2a–e, g, j).

Emeid compacta frequently contain growth marks, usually LAGs but also sometimes lamellar annuli that interrupt faster-growing parallel-fibred zones in the mid-cortex and that become increasingly dense and narrow towards the periosteum (Fig. 2a). Many of these growth marks are paired rather than single structures (Fig. 2b, e, j), a characteristic also shown by the non-avian theropod *Timimus hermani*¹⁸ and some extant ectothermic tetrapods¹. Growth marks are present in the cortex of only one sampled *Dinornis* (MNZ S 24365), in which the fibrolamellar compacta is interrupted by three pairs of narrow, densely lamellated annuli, the central pair of which both contain a distinct LAG (Fig. 2h). Moa growth marks follow the pattern of decreasing outward separation expected for true annual signals, and so can be used for skeletochronology, with consistently paired marks representing single annual events¹. No specimens with multiple growth marks show extensive endosteal resorption, which can remove evidence of earlier growth cycles, and so growth mark counts can be interpreted as accurate measures of the time taken for different moa species to reach skeletal maturity.

Characteristics of avian bone compacta have formerly been interpreted as clade-specific⁹, but histological diversity of primary cortical bone is now known to reflect differential growth rates rather than phylogenetic constraints ('Amprino's rule') in living ratites¹⁹ and other birds²⁰. Faster growth rates produce a less structured matrix, larger and more randomly distributed osteocytes, and an increased density of blood vessels with more complex network organization^{7,21}. The fibrolamellar compacta of *Dinornis* therefore indicates a markedly faster rate of cortical deposition during sub-adult development than does the largely lamellar-zonal compacta of emeids.

These developmental differences between the two moa families can be quantified by growth mark data. Although *Dinornis* took three years to reach adult body size, this is faster than the growth rate of the smallest emeid species, *Euryapteryx curtus*, which had a body mass of only 12–34 kg (ref. 16) but shows up to four growth marks in its outer cortex. Other emeids show even higher numbers of cortical growth marks, suggesting that these species had even longer sub-adult intervals. *Megalapteryx didinus* has up to five cortical growth marks, *Anomalopteryx didiformis* up to eight, and a *Euryapteryx geranoides* femur (CM SB282; Fig. 2j) shows nine sets of single, paired or grouped LAGs, suggesting that this species took at least nine years to reach adult body size. As currently recognized, the Emeidae is a paraphyletic family, with *Megalapteryx* representing the sister taxon to both *Dinornis* and the remaining emeid genera (Fig. 3a), so that the emeid rather than the dinornithid pattern of development might represent the plesiomorphic moa condition. *Dinornis* therefore appears to have attained its massive stature by accelerating its juvenile growth rate relative to that of emeids, rather than by extending its skeletal growth interval. This same developmental mechanism was also used to generate considerable size-differentiation between many dinosaur and pterosaur lineages^{2,22–24}.

Moa are phylogenetically nested within the extant ratite radiation^{25,26} (Fig. 3a). They were formerly²⁵ considered most closely related to kiwi (*Apteryx* spp.), the only members of the group found today in New Zealand. Kiwi are the smallest living ratites (250–550 mm high, 1.0–3.5 kg), and are obligate insectivores or carnivores rather than herbivores^{3,16}. Modern morphological and molecular studies²⁶ indicate that kiwi form part of a separate Australasian ratite clade that also contains cassowaries and emu,

but as part of the *K*-selected New Zealand avifauna that evolved in the absence of mammalian predators, they have a slow reproductive rate and prolonged immaturity, and might represent the closest living developmental analogue for moa. However, although kiwi do not reach sexual maturity until up to four years of age, they attain adult body weight at approximately 12 months (ref. 3). Other ratite species mature much more rapidly, and many species are able to lay fertile eggs within a year of hatching³. LAGs or annuli are unknown in living ratites^{2,4,9,17}, as these species apparently all develop too rapidly for annual growth marks to be deposited in their long bone compacta (Fig. 3b).

Extant bird species all show strictly determinate growth²⁷. Despite the absence of cortical growth marks in some emeids (*Emeus crassus*, *Pachyornis elephantopus* and *P. mappini*), and the intraspecific variation in growth mark number shown by other species, it is unlikely that ontogenetic rates varied greatly within moa. Previous studies^{2,5} have concluded that growth marks in endotherms are the result of inherited endogenous rhythms, but histological variation across the Dinornithiformes suggests instead that ornithurine osteogenesis might be phenotypically plastic, with growth marks in slow-growing taxa only deposited in response to particular conditions. This is supported by the observation that the most extensive avascular annuli are shown by *Megalapteryx didinus*, the habitat of which extended above the treeline (900–1,200 m elevation) in the Southern Alps¹⁶, and so it would have been exposed to more severe

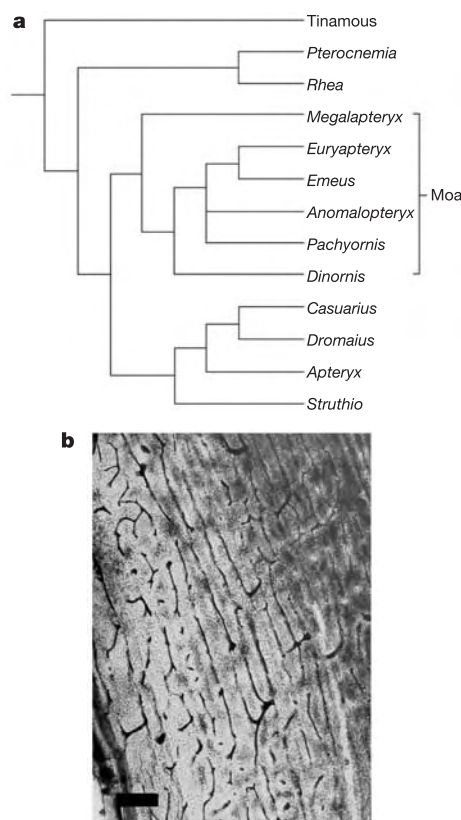


Figure 3 | Ratite phylogeny and histology. **a**, Cladogram showing the placement of moa within the extant ratite radiation, derived from morphological and molecular analyses^{16,26}. All moa genera other than *Dinornis* are currently assigned to the Emeidae¹⁶, making this taxon paraphyletic. **b**, Bright field photomicrograph of a transverse lapidary thin section through the tibiotarsus of a southern cassowary *Casuarii casuarii* (Smithsonian collections, USNM 429823), showing a fibrolamellar matrix similar to that of *Dinornis*, and characteristic of rapid osteogenesis. The section was taken through the diaphysis, near the distal metaphysis. Image provided courtesy of Peter Houde. Scale bar, 0.3 mm.

annual environmental fluctuations than lowland species. Polar bears and hibernating mammals also show LAGs in their bone compacta¹⁸, so histological evidence of arrested growth in response to marked seasonality might be widespread even in endotherms.

Variation in cortical structure and growth mark expression between different moa species—a single order of uncontroversially tachymetabolic ornithurines inhabiting broadly similar temperate conditions—suggests that physiological inferences for extinct avian or non-avian theropod groups made on the basis of such histological characters⁴ might be inappropriate. However, all moa showed considerably delayed maturity relative to other ratites, or indeed any extant birds. The slow reproductive cycle, reflecting a strongly K-selected life-history strategy that evolved in New Zealand's unique ecosystem, reveals unexpected flexibility in the ornithurine growth trajectory. Unfortunately, it would also have left moa extremely vulnerable to hunting by early human colonists²⁸, as has also been shown for mammalian representatives of the extinct Quaternary megafauna²⁹.

METHODS

Although different avian and non-avian theropod skeletal elements from the same individual can show considerable histological variation, femoral and tibiotarsal midshafts are the least remodelled and so provide the best record of growth processes through ontogeny^{4,30}. Histological analysis is restricted to moa samples showing little or no evidence of secondary remodelling. Femoral and tibiotarsal sections of *M. didinus* show a similar histological signal of multiple LAGs, indicating that these elements provide comparable ontogenetic data; comparison of tibiotarsal and tarsometatarsal sections of *P. elephantopus* shows that these elements are also histologically identical in moa.

Thin bone fragments (approximately 5 mm × 10 mm) cut from museum specimens were mounted on a frosted glass slide using a thermoplastic adhesive (Lakeside 70C). The free surface was then ground using a variable speed hand-held Dremel multi tool, with progress viewed using a Wild M8 stereozoom binocular microscope. The grinder was operated at a maximum speed of 20,000 r.p.m. when high stock removal aluminium oxide abrasive wheels or discs were used. Surfaces were finished using a felt polishing wheel operating at speeds of 18,000 r.p.m. A directed jet of compressed air was used to remove all fine dust before attaching the prepared surface to a second frosted slide using a cyanoacrylate ('super glue') adhesive, and repeating the grinding process until internal structures were clearly visible. All specimens were ground dry. Prepared slides have a removable glycerol-mounted coverslip to preserve the bone. Brightfield illuminated images were obtained using a Nikon Optiophot-2 biological microscope and a single-chip CCD RGB camera, and processed using AcQuis imaging software. Slides have been lodged at the National Museum of New Zealand Te Papa Tongarewa and Canterbury Museum.

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LETTERS

The initiation of liver development is dependent on Foxa transcription factors

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The specification of the vertebrate liver is thought to occur in a two-step process, beginning with the establishment of competence within the foregut endoderm for responding to organ-specific signals, followed by the induction of liver-specific genes. On the basis of expression and *in vitro* studies, it has been proposed that the Foxa transcription factors establish competence by opening compacted chromatin structures within liver-specific target genes¹. Here we show that Foxa1 and Foxa2 (forkhead box proteins A1 and A2) are required in concert for hepatic specification in mouse. In embryos deficient for both genes in the foregut endoderm, no liver bud is evident and expression of the hepatoblast marker alpha-fetoprotein (*Afp*) is lost. Furthermore, *Foxa1/Foxa2*-deficient endoderm cultured in the presence of exogenous fibroblast growth factor 2 (FGF2) fails to initiate expression of the liver markers albumin and transthyretin. Thus, Foxa1 and Foxa2 are required for the establishment of competence within the foregut endoderm and the onset of hepatogenesis.

The definitive endoderm is an epithelial sheet formed at the ventral side of the vertebrate embryo during gastrulation. Invagination of the endoderm at the anterior end of the embryo generates the ventral foregut, which ultimately gives rise to the liver, lung, thyroid and the ventral pancreas. The dorsal region of the definitive endoderm develops into the intestines and the dorsal bud of the pancreas. The specification and development of these domains are thought to be controlled by cell-autonomous factors such as transcriptional regulators, as well as by inductive or inhibitory signals from surrounding tissues. For example, *in vitro* explant studies have shown that FGF is sufficient to induce the differentiation of ventral foregut endoderm cells into hepatoblasts^{2–4}. It has been hypothesized, however, that the endoderm must first enter a stage in which competence to respond to FGF signalling is established. This model is based on the observation that regions of dorsal endoderm, which does not normally give rise to liver, can be induced to express the liver marker albumin if dissected between gestational days 8.5 and 11.5 and cultured in the presence of FGF; this competence is lost if the dorsal endoderm is isolated at embryonic day (E)13.5 or beyond, suggesting that factors required for competence are restricted to specific stages of embryonic development⁵. However, the factors required for the establishment of competence have not been identified.

The three members of the *Foxa* gene family of forkhead box-containing transcription factors (*Foxa1*, *Foxa2* and *Foxa3*) are candidate mediators of hepatic competence. These genes were initially cloned on the basis of their binding to regulatory regions of the transthyretin (*Ttr*) and α -1-antitrypsin genes^{6–8}, and they have since been shown to regulate the expression of a variety of regulatory and metabolic proteins expressed in the liver⁹. In the endoderm, the onset of *Foxa* gene expression precedes the induction of the hepatic programme by FGF signals. Furthermore, Foxa proteins are able to displace nucleosomes present in the regulatory region of the albumin

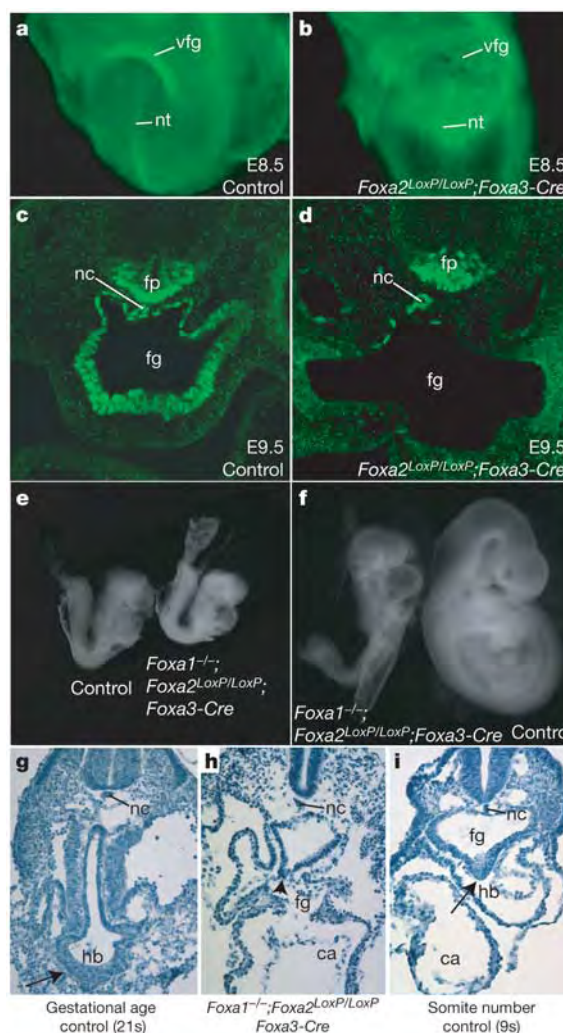


Figure 1 | The *Foxa1*^{-/-};*Foxa2*^{LoxP/LoxP};*Foxa3*-Cre mutant embryo. **a–d**, Endoderm-specific deletion of *Foxa2* was confirmed by whole-mount immunofluorescence staining of Foxa2 (green) in control and *Foxa2*^{LoxP/LoxP};*Foxa3*-Cre embryos at E8.5 (**a**, **b**) and E9.5 (**c**, **d**; axial sections). **e**, **f**, Control and *Foxa1*^{-/-};*Foxa2*^{LoxP/LoxP};*Foxa3*-Cre embryos at E8.5 (**e**) and E9.5 (**f**). **g–i**, Hepatic buds were evident (arrows) in axial sections of age- and somite number-matched control embryos (**g**, **i**), but not in the *Foxa1*^{-/-};*Foxa2*^{LoxP/LoxP};*Foxa3*-Cre embryo (arrow in **h**). Abbreviations: ca, cardiac mesoderm; fg, foregut; fp, floor plate; hb, hepatic bud; nc, notochord; nt, neural tube; s, somites; vfg, ventral foregut.

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gene before the gene becomes activated, but other transcription factors that bind to this region are unable to do so^{10–13}. *Foxa2* binding can reverse chromatin-mediated repression of alpha-fetoprotein (*Afp*) gene transcription *in vitro*¹⁴. Thus, the timing of *Foxa1–3* expression in the early endoderm and the ability of these proteins to modify chromatin raise the possibility that the *Foxa* genes are responsible for the establishment of competence within the foregut endoderm¹. We set out to test this hypothesis *in vivo* using a genetic approach.

We have previously explored the possibility that individual members of the *Foxa* family are required for liver development. Mice homozygous for targeted inactivation of the *Foxa1* gene die during the first two weeks of post-natal life, but their livers appear histologically normal¹⁵. Mice bearing null mutations in the *Foxa3* gene also have apparently normal livers¹⁶. Because mouse embryos lacking *Foxa2* die shortly after gastrulation owing to node and notochord defects^{17,18}, it was necessary to develop a mouse model in which expression of *Foxa2* is maintained in the axial mesoderm but is abrogated in the foregut endoderm. For this purpose, we engineered an endoderm-specific deletion of *Foxa2* using the Cre/loxP recombination system, in which the *Cre* gene was placed under the control of the *Foxa3* promoter. As shown in Fig. 1a–d, *Foxa2* expression is maintained in the notochord and floor plate of the neural tube, but is deleted in the foregut endoderm from E8.5. Nevertheless, in *Foxa2^{LoxP/LoxP};Foxa3-Cre* mice hepatic induction and growth occur normally¹⁹. Therefore, genetic analysis has shown that single deletions of *Foxa1*, *Foxa2* or *Foxa3* do not prevent the establishment of competence in the foregut endothelium or hepatic development.

The similarities in structure and DNA-binding specificity shared by *Foxa1–3* suggest that they can compensate for the loss of one

family member. We therefore hypothesized that losing combinations of *Foxa* genes in the developing endoderm would reveal a requirement for these factors in early liver development. We first derived mice lacking both *Foxa1* and *Foxa3*, and found that these mice resembled *Foxa1* knockout (*Foxa1^{-/-}*) mice and developed grossly normal livers (data not shown). Next we bred mice in which both *Foxa1* and *Foxa2* were deleted at the onset of hepatic specification. To achieve this, we mated *Foxa1^{+/-};Foxa2^{+LoxP};Foxa3-Cre* mice with *Foxa1^{+/-};Foxa2^{LoxP/LoxP}* mice. One-sixteenth of the offspring from this mating were expected to have the genotype *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre*, and thus lack both *Foxa1* and *Foxa2* in the foregut endoderm.

However, no embryos were born bearing the double mutation, and analysis of 219 mid-gestation embryos revealed no such embryos beyond E10. However, just after hepatic induction on E8.5, *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos were found at the expected mendelian ratio (1/16). At E8.5, these embryos appeared indistinguishable from their control littermates and from embryos in which only *Foxa1* or *Foxa2* had been deleted (Fig. 1e and data not shown). At E9.5, most of the *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos were smaller (~9 somites) compared with their control littermates (~21 somites), and except in a single case, were not turned (Figs 1f and 2d). Although the *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos had fewer somites than control littermates, gross development of the head folds and limb buds was normal.

Microscopic examination of sectioned *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos revealed no evidence of hepatic bud formation, whereas hepatoblasts were present in control littermates in the ventral foregut (Fig. 1g, h). Because of the difference in somite numbers between *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* and littermate

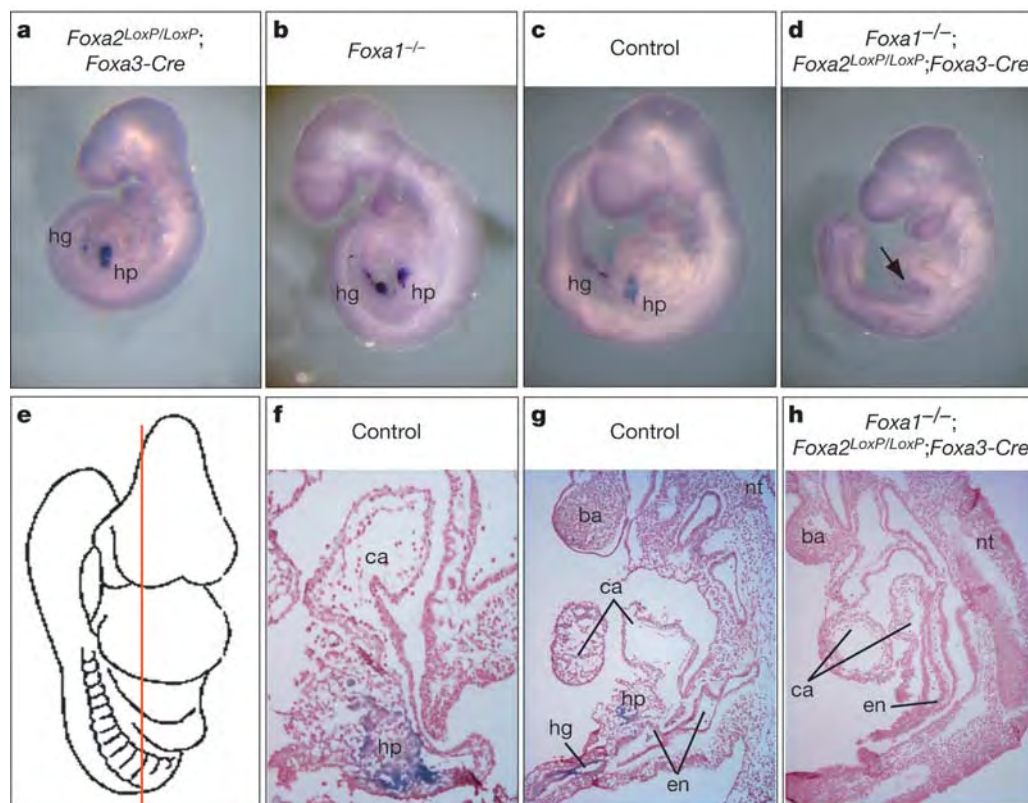


Figure 2 | *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos fail to initiate the hepatic programme. **a–d**, **f–h**, Whole-mount RNA *in situ* hybridization analysis of *Afp* in E9.5 *Foxa2^{LoxP/LoxP};Foxa3-Cre* (**a**), *Foxa1^{-/-}* (**b**), control (**c**, **f**, **g**) and *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* (**d**, **h**) embryos. The arrow in **d** indicates the expected location of the hepatic primordium. **f–h**, Sagittal

sections of the control and *Foxa1^{-/-};Foxa2^{LoxP/LoxP};Foxa3-Cre* embryos in the plane depicted in **e**. Abbreviations: ba, branchial arch; ca, cardiac mesoderm; en, endoderm; hg, hindgut; hp, hepatic primordium; nt, neural tube.

embryos, comparisons were also made to controls matched for somite number, and in these controls liver bud formation could also be seen (Fig. 1i). Thus, the lack of liver induction in *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* mice was not simply due to a delay in development. Other structures, including the axial mesoderm, were formed properly in the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryos, suggesting that the hepatic defect is caused by the absence of *Foxa1* and *Foxa2* in the foregut endoderm, and not by abnormalities in the surrounding tissues (Fig. 1g–i).

To further investigate the defects in the hepatic primordium of the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryos, we performed whole-mount RNA *in situ* hybridization to detect *Afp* expression. *Afp* mRNA was detected in the hepatic primordium and hindgut of control embryos as well as embryos carrying single deletions of *Foxa1* or *Foxa2* (Fig. 2a–c). Microscopic examination of the stained embryos showed *Afp* expression in hepatoblasts adjacent to the ventral foregut endoderm (Fig. 2f, g). In contrast, there was no *Afp* expression in the ventral foregut of the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryos, suggesting a failure of hepatic specification (Fig. 2d, h). However, these data leave open the possibility that induction of the hepatic programme does occur in the absence of *Foxa1* and *Foxa2*, but that there is a subsequent failure of the nascent hepatoblasts to proliferate, as has been observed in *Hex*^{-/-} mice²⁰.

To test the hypothesis that hepatic specification is fully abrogated by the loss of *Foxa1* and *Foxa2*, we used the *in vitro* explant culture system developed in ref. 3. In this system, the earliest steps of hepatic

induction can be modelled *in vitro* by co-culture of foregut endoderm with FGF, allowing us to test the competency of the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* endoderm. Ventral foregut endoderm was dissected from embryos at the 4–6 somite stage and was cultured for 48 h in the presence of FGF2 and heparan sulphate, as it has been shown that this treatment is sufficient to induce hepatic differentiation in the absence of the cardiac mesoderm²¹. Consistent with these prior studies, expression of the hepatoblast markers albumin and transthyretin was induced in control embryos and in those lacking only *Foxa1* or *Foxa2*. In contrast, expression of these genes was nearly undetectable in *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryos (Fig. 3a, b). These data show that failure of hepatic specification in the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryo is due to a loss of competence in responding to normal inductive signalling by FGF.

In summary, these results identify *Foxa1* and *Foxa2* as essential for hepatic specification. Although mutations in endodermal transcription factors such as *Hex*^{20,22,23}, *HNF4α* (ref. 24), *HNF1β* (*Tcf2*; ref. 25) and *HNF6* (*Onecut1*; ref. 26) result in abnormalities in liver development, in each case the defects occur at stages after initial hepatic specification. The *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* mouse is the first vertebrate model in which the specification of an endoderm-derived organ does not occur. These findings also provide genetic support for the competency model, in which *Foxa* proteins function as 'pioneer' proteins to open compacted chromatin in regulatory regions of liver-specific genes. The function of *Foxa* genes in liver specification provides a model for the molecular control of organogenesis, and future studies can be directed towards investigating the degree to which similar mechanisms are used in the development of other endoderm-derived organs.

METHODS

Whole-mount RNA *in situ* hybridization. RNA *in situ* hybridization was performed using a digoxigenin (DIG)-UTP (Roche) labelled antisense RNA probe as described²⁷. Signal was detected using an alkaline-phosphatase-conjugated anti-DIG antibody and NBT/BCIP substrate (Roche). The antisense *Afp* probe was synthesized using nucleotides 1448–1854 of the mouse *Afp* complementary DNA. Sectioned embryos were counterstained with nuclear fast red.

Whole-mount immunostaining. Whole-mount immunofluorescent detection of *Foxa2* was performed on E8.5 and E9.5 embryos as described¹⁹.

Embryo explant culture. Explants were cultured in microwells (Nunc) coated with type I collagen (Collaborative Biomedical Products) in DMEM medium supplemented with 10% calf serum (Hyclone) at 37 °C in the presence of 5% CO₂. Recombinant human FGF2 (Sigma; 5 ng ml⁻¹) and 50 ng ml⁻¹ heparan sulphate (Sigma) were added to all samples.

Histology. Embryos were fixed in 4% paraformaldehyde overnight at 4 °C, washed with PBS, incubated in 30% sucrose, then embedded in tissue freezing medium (Tissue Tek) and serially sectioned in a cryostat at 8-μm thickness. Tissues were stained with haematoxylin (Zymed).

RNA extraction and quantitative polymerase chain reaction. RNA was extracted from explants using the RNeasy micro kit (Qiagen). Reverse-transcription was primed with oligo d(T). A Stratagene Mx4000 real-time PCR machine was used for the quantitative PCR analysis. Conditions and primer concentrations suggested by the SYBR Green assay protocol were followed. Primer sequences are available upon request.

Mice. Mice were kept on a mixed background. The derivation of the *Foxa1* null mice and *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* mice has been described elsewhere^{15,19}. A total of 458 embryos were analysed, including 14 *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* mutants.

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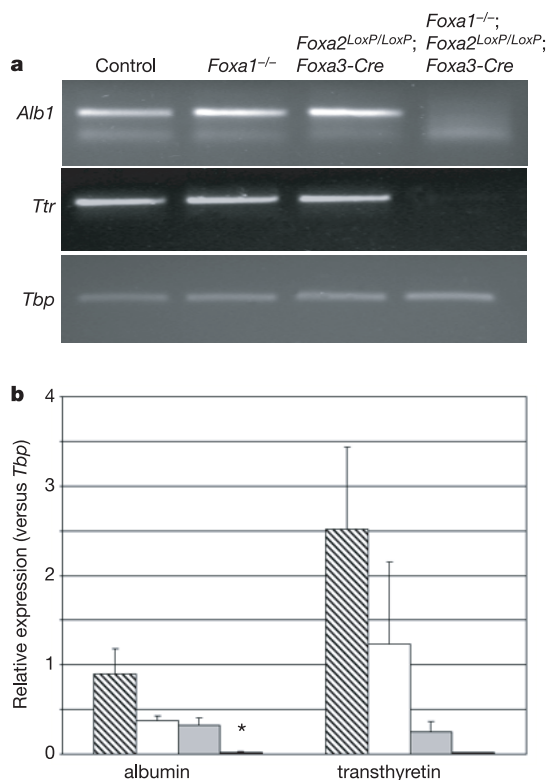


Figure 3 | *In vitro* activation of the hepatic programme by FGF is abrogated in *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* embryos. **a**, PCR with reverse transcription (RT–PCR) of albumin (*Alb1*), transthyretin (*Ttr*) and TATA-box binding protein (*Tbp*) messenger RNA in ventral endoderm cultured from the indicated embryos. **b**, Quantitative RT–PCR analysis of albumin and transthyretin mRNA in control (striped bars; *n* = 6), *Foxa1*^{-/-} (white bars; *n* = 2), *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* (grey bars; *n* = 3), and *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* (black bars; *n* = 2 for albumin and *n* = 1 for transthyretin). Each bar represents mean ± s.e.m. There is no error bar for transthyretin expression in the *Foxa1*^{-/-}; *Foxa2*^{LoxP/LoxP}; *Foxa3-Cre* cultured ventral endoderm. Asterisk denotes a *P* value of <0.05.

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LETTERS

A Pax3/Pax7-dependent population of skeletal muscle progenitor cells

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During vertebrate development, successive phases of embryonic and fetal myogenesis lead to the formation and growth of skeletal muscles¹. Although the origin and molecular regulation of the earliest embryonic muscle cells is well understood², less is known about later stages of myogenesis. We have identified a new cell population that expresses the transcription factors Pax3 and Pax7 (paired box proteins 3 and 7) but no skeletal-muscle-specific markers. These cells are maintained as a proliferating population in embryonic and fetal muscles of the trunk and limbs throughout development. Using a stable green fluorescent protein (GFP) reporter targeted to Pax3, we demonstrate that they constitute resident muscle progenitor cells that subsequently become myogenic and form skeletal muscle. Late in fetal development, these cells adopt a satellite cell position characteristic of progenitor cells in postnatal muscle. In the absence of both Pax3 and Pax7, further muscle development is arrested and only the early embryonic muscle of the myotome forms. Cells failing to express Pax3 or Pax7 die or assume a non-myogenic fate. We conclude that this resident Pax3/Pax7-dependent progenitor cell population constitutes a source of myogenic cells of prime importance for skeletal muscle formation, a finding also of potential value in the context of cell therapy for muscle disease.

During the onset of skeletal myogenesis in the embryo, Pax3 is required for the survival of the ventro-lateral dermomyotome, the part of the somite that gives rise to hypaxial body^{3,4} and limb⁵ musculature. Pax3 is also implicated in the determination of myogenic cell fate, acting through MyoD⁶. In the absence of the myogenic regulatory factors MyoD, Myf5 and Mrf4 (ref. 7), skeletal muscle does not form and no myogenic cells are present⁸. Most of the functions of Pax3 can be replaced by its paralogue Pax7 (ref. 9). However, Pax7, which is also expressed in the somite, is only essential for myogenesis after birth, when it is expressed in satellite cells (the progenitors of adult skeletal muscle)^{10,11}.

Pax3 is first expressed in the presomitic mesoderm and this expression is maintained in the somitic epithelium of the dermomyotome^{12–15} (Fig. 1a). However, at embryonic day (E)10.5, Pax3 is also detected (by immunohistochemistry) in the myotome, the first skeletal muscle to form¹⁶ (Fig. 1b, c). In less mature posterior somites, this expression is first observed in a few cells lying under the epithelial dermomyotome (Fig. 1a), whereas in more mature anterior somites, Pax3-expressing cells are found throughout the myotome (Fig. 1b, c). The number of Pax3-positive (Pax3⁺) myotomal cells increases as the central dermomyotome loses its epithelial structure (Fig. 1d (middle and right panels) and e), suggesting that they arise directly from the central dermomyotome. Similar results were observed for Pax7, which is first expressed in the central dermomyotome^{3,17} (Supplementary Fig. S1a) and then co-localizes with Pax3-positive cells in the myotome (Fig. 1e, right panel), where about 87% of cells are

Pax3⁺Pax7⁺, 10% are Pax3⁺ only, and 3% are Pax7⁺ only. Recent experiments in the chick embryo show that Pax3⁺Pax7⁺ cells in the myotome derive from the dermomyotome²⁵.

At E10.5, expression of the myogenic determination factor Myf5, visualized as β -galactosidase (β -gal) from a *Myf5^{nLacZ}* allele, is seen in the lips of the dermomyotome¹⁶, which also express high levels of Pax3 (arrows in Fig. 1d). In contrast, most Pax3⁺Pax7⁺ cells in the myotome do not co-express Myf5 (about 93% are Myf5[−]) (Fig. 1d and Supplementary Fig. S1a). This lack of co-expression is also seen for other markers of cell engagement in the myogenic programme, such as desmin (Supplementary Fig. S1b) or MyoD (Fig. 1f). No co-expression was detected for sarcomeric myosin heavy chain (MyHC) (Fig. 1g), which marks differentiated cells.

The Pax3⁺Pax7⁺ population shows labelling with a mitotic marker (Fig. 1h), indicating the presence of dividing cells. We examined the proliferation of this population compared to the myogenic cells in the myotome, using co-localization with cyclin A, a marker of S and G2 phases (which represent about 50% of the cell cycle length). Data presented in Fig. 1m–o, bottom panels and quantified in Fig. 1p show that about 96% of the Pax3⁺ cells in the dermomyotome are dividing, as are 81% of the Pax3⁺Pax7⁺ progenitors in the myotome. In contrast, less than 20% of the MyoD⁺ cells in the myotome are proliferating (Fig. 1o, bottom panel) and only 8% of the Myf5(β -gal)⁺ cells are proliferating (Fig. 1n, bottom panel), although this lower figure for Myf5 probably reflects β -gal stability from the *Myf5^{nLacZ}* allele with labelling of some differentiating muscle cells. Of the total number of cyclinA-positive cells in the myotome, 76% are Pax3⁺ (Fig. 1m, lower panel). We conclude that the Pax3⁺Pax7⁺ cells constitute the main proliferating population of the myotome. Cells that are not detected as proliferating might reflect heterogeneity in the cell population, or might correspond to cells that are progressing into the myogenic programme, but for which Pax3 expression is still detectable.

Analysis of skeletal muscle masses at later stages shows that the Pax3⁺Pax7⁺ population persists and continues to be distinct from cells that have entered the myogenic programme. MyoD, which marks the myogenic cells, does not co-localize with Pax7, as determined either by staining with an antibody recognizing Pax7 (Fig. 1i, right panel) or by a more sensitive assay using an antibody to β -gal generated from the *Pax7^{LacZ}* allele¹⁸ (Fig. 1j, right panel). Similar results were obtained at all stages examined (E11.5–E17.5; Figs 2, 3, Supplementary Fig. S1 and data not shown). At later stages, Pax3⁺Pax7⁺ cells are also actively dividing (Fig. 1k, right panel). They are also clearly distinct from connective tissue, which is labelled using an antibody against T-cell factor 4 (TCF4) (ref. 19; Fig. 1l, right panel).

Having identified a novel Pax3⁺Pax7⁺ population of proliferating cells distinct from the myogenic cells of skeletal muscle, we then investigated whether this population contributes to the growth of

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this tissue. We took advantage of the fact that the products of different reporter genes targeted to the *Pax3* and *Pax7* loci¹⁸ have different stabilities (Fig. 2s and Supplementary Fig. S2). GFP, encoded by the reporter sequence that we targeted to *Pax3* (F.R. and M.B., unpublished data), is more stable than β -gal generated from the *Pax3*^{IRESnLacZ/+} or *Pax7*^{LacZ/+} alleles^{18,20}, which has comparable stability to the endogenous proteins (Supplementary Fig. S2). Most (93%) of β -gal⁺ cells detected in trunk muscles of *Pax7*^{LacZ/+} mice at E13.5 or E15.5 do not co-express desmin (Fig. 2a–d, j–l), but they do co-localize with the GFP⁺ cells generated from the *Pax3*^{GFP/+} allele (Fig. 2e). However, there are additional GFP⁺ cells that are not

β -gal⁺ (Fig. 2e), and all of these co-localize with desmin at E13.5 (Fig. 2f). Similar results were obtained at E15.5, when multinucleated muscle fibres are clearly both GFP- and desmin-positive (Fig. 2m–o), in contrast to some individual cells labelled only with GFP (arrows in Fig. 2m–o). In addition, nearly all (87%) of the *Pax7*⁺ cells are dividing, as shown by co-expression of Ki67, which marks cycling cells (Fig. 2g–i), whereas only 9% of the GFP⁺/*Pax7*⁺ cells co-express Ki67 (data not shown). At this stage, 89% of cycling cells located within the muscle masses express Pax7, demonstrating that the *Pax3*⁺/*Pax7*⁺ progenitors constitute the main proliferating cell population (Fig. 2g–i).

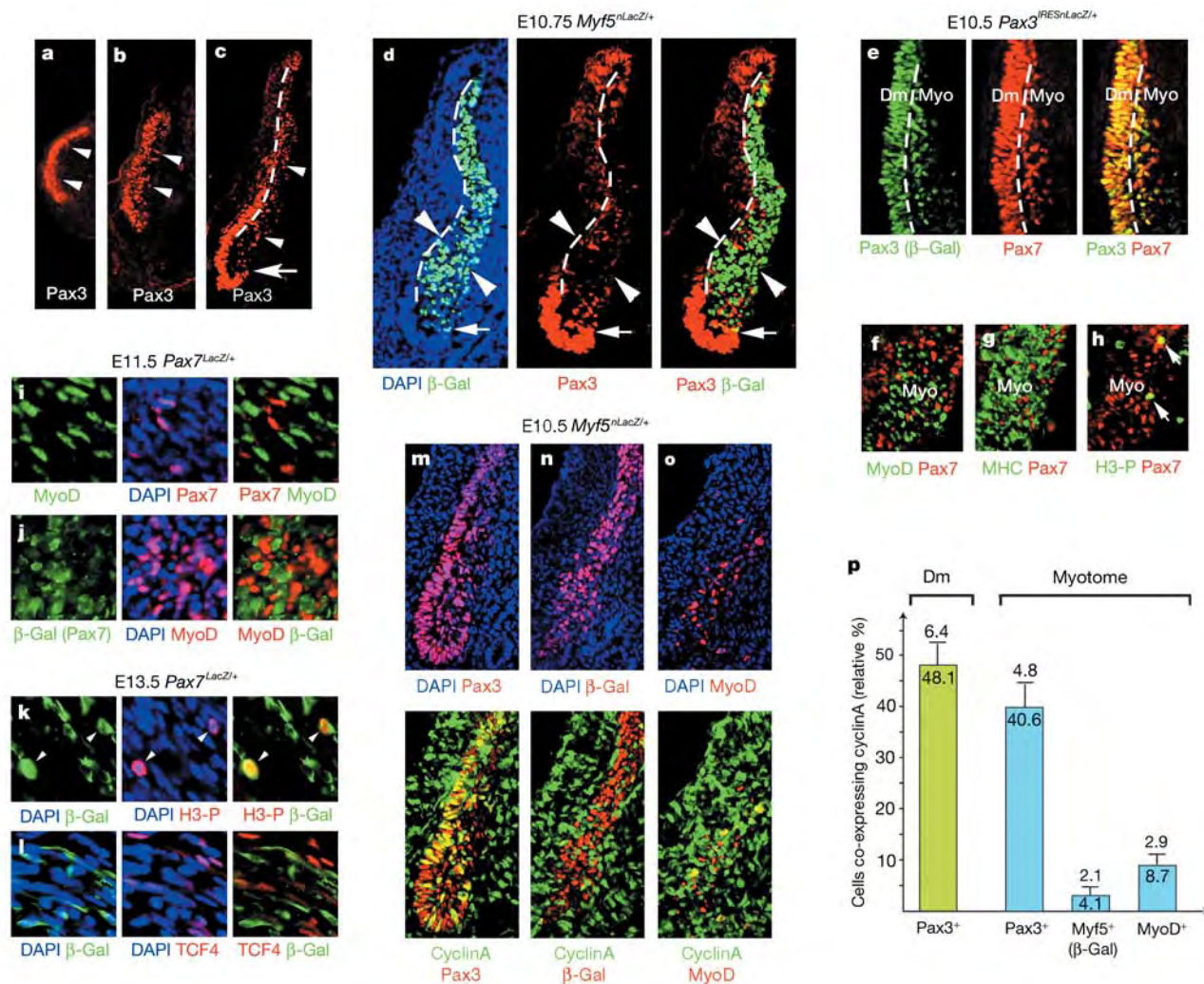


Figure 1 | Pax7 and Pax3 label a distinct cell population present in embryonic skeletal muscle. Where appropriate, detected proteins in Figs 1–4 are written underneath individual panels in the colour corresponding to the fluorescent label or stain. **a–c**, Transverse sections at different somite levels, stained with a Pax3-specific antibody. Arrowheads point to labelled cells in the myotome. In **c**, a dotted line shows the separation between the dermomyotome (on the left) and the myotome. At this stage, the dermomyotome is disintegrating in the central region while the epithelial structure is maintained hypaxially (arrow). **d**, Co-immunohistochemistry on transverse sections of thoracic somites (36-somite stage) from *Myf5*^{nLacZ/+} embryos at E10.75, using antibodies recognizing Pax3 and β -gal from the *Myf5*^{nLacZ/+} allele²⁴. The myogenic markers do not co-localize with Pax3. Arrowheads indicate the width of the myotome. The arrow indicates the hypaxial extremity of the dermomyotome. A dotted line shows the separation between the dermomyotome (on the left) and the myotome. **e**, Co-immunohistochemistry on transverse sections of hypaxial thoracic somites from a *Pax3*^{IRESnLacZ/+} embryo at E10.5, using antibodies

recognizing β -gal and Pax7, showing co-localization in the myotome. Dm, dermomyotome, Myo, myotome. **f–h**, Co-immunohistochemistry on transverse sections of hypaxial thoracic somites from embryos at E10.5, using antibodies recognizing Pax7 and MyoD (**f**), MyHC (MHC) (**g**) or phosphorylated histone H3 (H3-P, **h**), showing that Pax7⁺ cells are dividing. **i–l**, Co-immunohistochemistry on transverse sections of E11.5 shoulder muscle (**i**, **j**) and hypaxial trunk muscle (**k**, **l**) from *Pax7*^{LacZ/+} embryos at E13.5 using antibodies directed against MyoD and Pax7 (**i**), MyoD and β -gal (**j**), H3-P and β -gal (**k**), and TCF4 and β -gal (**l**), showing that Pax7⁺ cells are dividing and independent of connective tissue. **m–o**, Co-immunohistochemistry on transverse sections of trunk somites from *Myf5*^{nLacZ/+} embryos at E10.5 using antibodies recognizing Pax3 (**m**), β -gal (**n**), MyoD (**o**) and cyclin A (bottom panels in **m–o**). DAPI staining is shown in the top panels of **m–o**. The hypaxial region is shown with Pax3-positive cells expressing cyclin A, whereas this is the case for only a few *Myf5*(β -gal)⁺ or *MyoD*⁺ cells. **p**, Quantification of ≥ 6 sections from at least two different embryos as shown in **m–o**. Error bars indicate standard deviation.

These observations strongly suggest that the dividing $Pax3^{+}Pax7^{+}$ cells provide a major source of myogenic cells for the formation of skeletal muscle fibres. When the $Pax3^{GFP/+}$ line is crossed with $Myf5^{nLacZ/+}$ mice, all β -gal⁺ cells are also GFP⁺ (Fig. 2p–r). Identical results were obtained with GFP and MyoD (Supplementary Fig. S1g–i and data not shown).

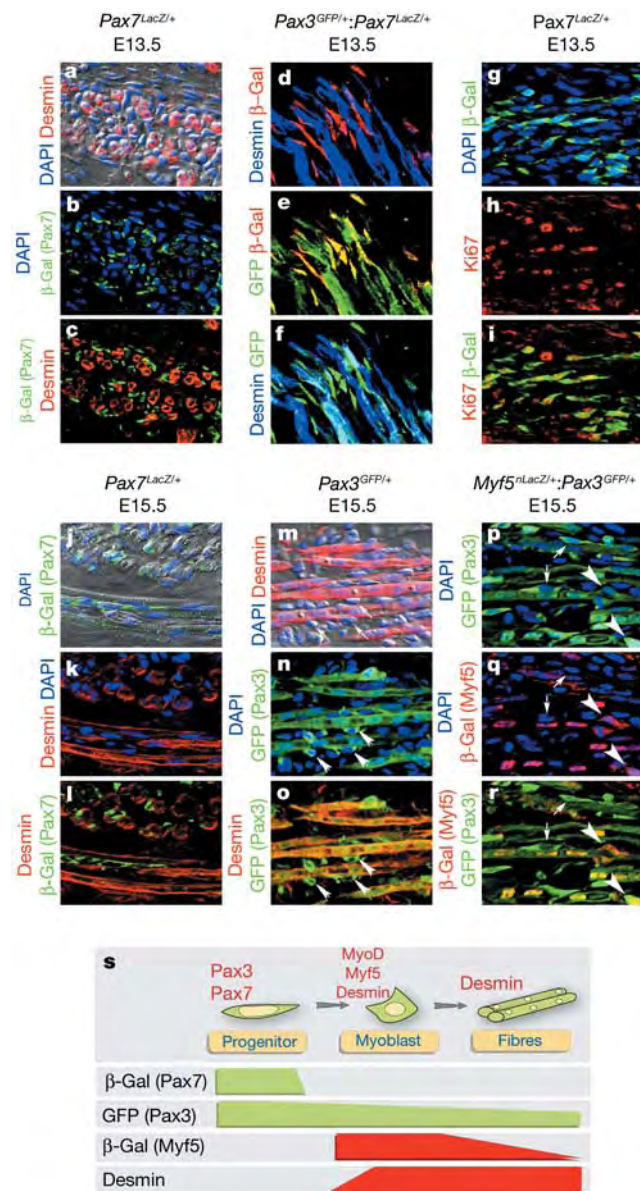


Figure 2 | $Pax3^{+}Pax7^{+}$ cells contribute to skeletal muscle. a–i, Co-immunohistochemistry at E13.5 on transverse sections from hypaxial trunk muscle of $Pax7^{LacZ/+}$ (a–c, g–i) or $Pax3^{GFP/+}; Pax7^{LacZ/+}$ (d–f) embryos, using antibodies directed against desmin (a, c, d, f), β -gal (b–e, g, i), or Ki67 (h, i). Phase-contrast image shown in a, GFP fluorescence in e, f and DAPI staining in a, b, g. j–r, Co-immunohistochemistry at E15.5 on transverse sections from hypaxial trunk muscle of $Pax7^{LacZ/+}$ (j–l), $Pax3^{GFP/+}$ (m–o) or $Myf5^{nLacZ/+}; Pax3^{GFP/+}$ (p–r) embryos, using antibodies directed against desmin (k–m, o), β -gal (j, l, q, r) or GFP (n–p, r). Phase-contrast images are shown in j, m, and DAPI staining is shown in j, k, m, n, p, q. GFP⁺desmin[−] cells and GFP⁺ β -gal[−] cells are indicated by white arrows in m–r. GFP⁺ β -gal⁺ myogenic cells are shown with arrowheads in p–r. s, Schematic showing the endogenous expression of Pax3 and Pax7 in resident muscle progenitor cells, the expression of Myf5 and MyoD in muscle myoblasts, and desmin in myoblasts and muscle fibres. This expression is compared with the relative expression of the LacZ, GFP and nLacZ reporter proteins generated from, $Pax7^{LacZ}$, $Pax3^{GFP}$ and $Myf5^{nLacZ}$ alleles, respectively.

We then investigated whether our findings on trunk muscles could be generalized to the limb, where the myogenic cells are derived from a progenitor cell population that migrates from the somites^{14,15}. These cells express Pax3 but not Pax7, which is upregulated at E11 in the mouse embryo⁹. At E13.5, we found that cells expressing $Pax7^{LacZ/+}$ are distinct from the desmin-positive muscle cells of the limb (Supplementary Fig. S1c–f). Analysis of $Pax3^{GFP/+}; Pax7^{LacZ/+}$ embryos at this stage also showed that all the Pax7⁺ cells express GFP (Supplementary Fig. S1d–f) and that all the MyoD⁺ myogenic cells, which do not express Pax7, are marked by GFP (Supplementary Fig. S1g–l). We conclude that the $Pax3^{+}Pax7^{+}$ cells constitute a novel compartment of resident muscle progenitor cells that contribute to muscle growth during development both in the limbs and trunk.

By E15.5, cells that are Pax3⁺ but Myf5(β -gal)[−] are located along, and in close contact with, the muscle fibres (Fig. 2p–r). Between E16.5 and E18.5 a basal lamina, marked by laminin expression, forms around the muscle fibres, including the associated Pax3⁺Pax7⁺ cells (Fig. 3a–o and Supplementary Fig. S1m–o). This location, under the basal lamina and in close proximity to the muscle fibre, is characteristic of satellite cells, which are the progenitor cells of postnatal skeletal muscle²¹ and have been shown to originate from the somite in the chick embryo²². In the mouse, these cells express Pax7 (ref. 10), and in a subset of skeletal muscles also express Pax3 (F.R. and M.B., unpublished data). We found that $Pax3^{GFP/+}$ cells become embedded under the basal lamina by E18.5 (Fig. 3m–o and Supplementary Fig. S1m–o) and are still detectable during postnatal growth (Fig. 3p–r). We suggest that the resident muscle progenitor cells present in embryonic and fetal muscle later constitute the satellite cell

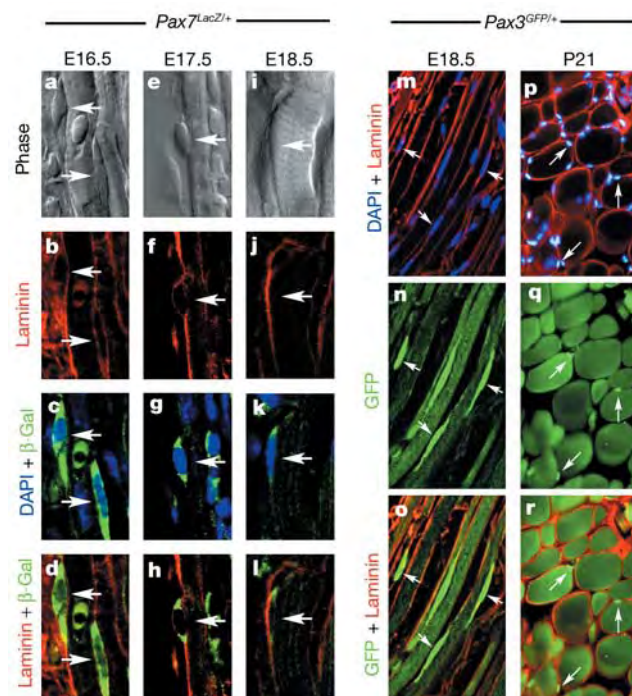


Figure 3 | $Pax3^{+}Pax7^{+}$ progenitors adopt a satellite cell position from late fetal stages. a–l, Co-immunohistochemistry on hypaxial trunk muscle from $Pax7^{LacZ/+}$ fetuses at E16.5 (a–d), E17.5 (e–h) and E18.5 (i–l), using DAPI staining (c, g, k) and antibodies directed against laminin (b, d, f, h, j, l) and β -gal (c, d, g, h, k, l). Phase-contrast images shown in a, e, i. β -gal⁺ cells become embedded in the basal lamina and adopt satellite cell positions (indicated with white arrows). m–r, Co-immunohistochemistry on hypaxial trunk muscle from $Pax3^{GFP/+}$ embryos at E18.5 (m–o) or three-week-old (P21) mice (p–r) using DAPI staining (m, p) and antibodies directed against laminin (m, o, p, r) and GFP (n, q, r). White arrows indicate GFP-positive cells located in a satellite cell position.

population responsible for postnatal muscle growth and regeneration.

As previously reported, *Pax7* mutants show no overt muscle defect during development^{9,18}. In contrast, in the trunk, *Pax3* mutant embryos display somite truncations²⁰ with loss of the hypaxial dermomyotome (Fig. 4b,e), resulting in the reduction and disorganization of hypaxial trunk musculature³ (Fig. 4h, arrowhead). The somites of *Pax3/Pax7* double mutants are similar to those of *Pax3* mutants until E10.5 (Fig. 4b–c), with initial formation of skeletal muscle in the myotome (Supplementary Fig. S3a, b) under the control of the myogenic regulatory factors *Myf5* (ref. 6) and/or *Mrf4* (ref. 7).

However, as development proceeds, somites become more severely affected and loss of *Pax3*⁺*Pax7*⁺ cells is observed (Fig. 4f). Subsequently, most skeletal muscles of the trunk, such as those of the overlying body wall (Fig. 4i), are severely compromised. By E13.5, only a few muscle fibres are detectable (Fig. 4l), and at E11.5 there is already a marked deficit of differentiated muscle cells (Fig. 4o). *Pax3* (and *Pax7* when substituted for *Pax3*, ref. 9) assures the survival of early myogenic progenitor cells in the hypaxial dermomyotome⁴. By E11.5, no further apoptosis is observed in the *Pax3* mutant, whereas in the *Pax3/Pax7* double mutant cell death still occurs (lower panels, Fig. 4m–o).

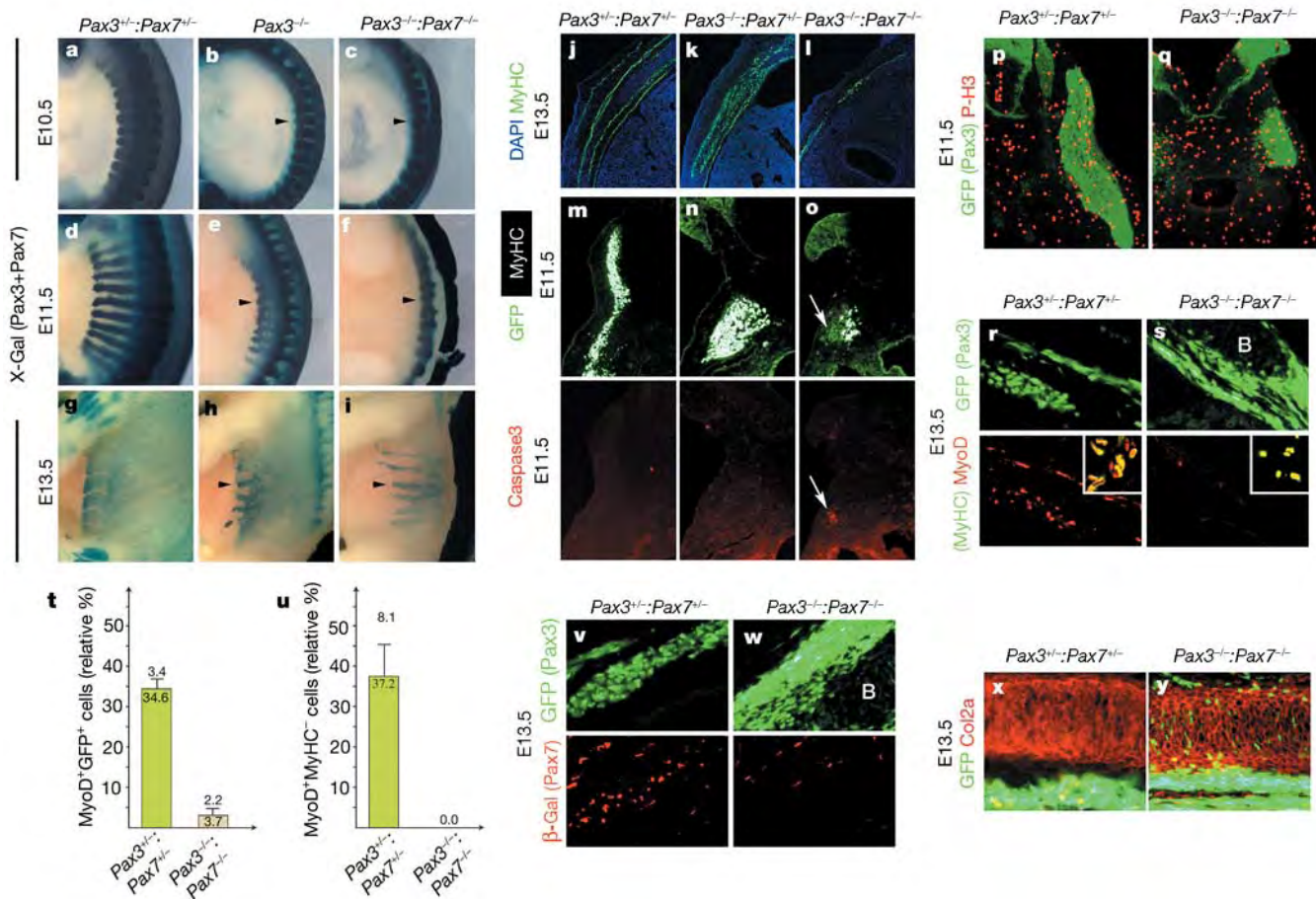


Figure 4 | Major myogenic defects in *Pax3/Pax7* double mutant embryos. **a–i**, X-gal staining of thoracic somites at E10.5 (**a–c**) and E11.5 (**d–f**) and of thoracic muscles at E13.5 (**g–i**) of *Pax3*^{nLacZ/+}*Pax7*^{LacZ/+} (*Pax3*^{+/+}*Pax7*^{+/+}, **a, d, g**), *Pax3*^{nLacZ/nLacZ} (*Pax3*^{-/-}, **b, e, h**) or *Pax3*^{nLacZ/nLacZ}*Pax7*^{LacZ/LacZ} (*Pax3*^{-/-}*Pax7*^{-/-}, **c, f, i**) embryos. Arrowheads indicate somite and trunk muscle defects. **j–l**, Immunohistochemistry on transverse sections of body wall muscle of *Pax3*^{GFP/+}*Pax7*^{LacZ/+} (*Pax3*^{+/+}*Pax7*^{+/+}, **j**), *Pax3*^{GFP/GFP}*Pax7*^{LacZ/+} (*Pax3*^{-/-}*Pax7*^{+/+}, **k**) or *Pax3*^{GFP/GFP}*Pax7*^{LacZ/LacZ} (*Pax3*^{-/-}*Pax7*^{-/-}, **l**) embryos at E13.5, using DAPI staining and an antibody against MyHC. Loss of skeletal muscle is seen in the double mutant. **m–o**, Co-immunohistochemistry on transverse sections of thoracic somites of E11.5 embryos as in **j–l**, using antibodies recognizing MyHC (top panels) and the activated form of caspase 3, which labels apoptotic cells (bottom panels). Endogenous GFP fluorescence is detectable above background and co-localizes with active caspase-3 (arrow in **o**), indicating that, in contrast to the *Pax3* mutant embryos, apoptosis is maintained in the *Pax3/Pax7* double mutants at E11.5. **p–y**, Co-immunohistochemistry (**p–s, v–y**) and histograms (**t, u**) using *Pax3*^{GFP/+}*Pax7*^{LacZ/+} (*Pax3*^{+/+}*Pax7*^{+/+}) or *Pax3*^{GFP/GFP}*Pax7*^{LacZ/LacZ} (*Pax3*^{-/-}*Pax7*^{-/-}) embryos. **p, q**, Transverse sections of thoracic somites from E11.5 embryos using antibodies recognizing GFP and phosphorylated histone H3 (P-H3), which labels mitotic cells. No proliferation defects are observed in the double mutant

embryos. **r, s**, Transverse sections of embryos at E13.5 using antibodies recognizing GFP (top panels), MyoD (bottom panels) and MyHC (insets in bottom panels). Insets show the co-localization (in yellow) of MyoD and MyHC in the control and *Pax3/Pax7* double mutants. There are no undifferentiated MyoD⁺/MyHC⁺ cells in the double mutants. **t**, Quantification of the number of GFP⁺ cells co-expressing MyoD in embryos shown in **r, s** (≥ 6 sections from 2 independent embryos in each case). **u**, Quantification of the number of MyoD⁺ cells co-expressing MyHC. In contrast to the control embryos, in which 37% of the MyoD⁺ cells are not terminally differentiated, all the MyoD⁺ cells co-express the differentiation marker MyHC in the double mutant embryos, demonstrating that at this stage no MyoD⁺ myogenic cells are contributing to muscle growth. Standard deviations are indicated on the histograms. **v, w**, Transverse sections of E13.5 embryos at the level of the rib, which is normally adjacent to intercostal muscle, using antibodies recognizing GFP (top panels) and β -gal (bottom panels), showing that only cells marked with the stable GFP reporter are still present. In **w** (top panel), some GFP⁺ cells localize in the bone (B) of double mutant embryos. **x, y**, Transverse sections in E13.5 embryos at the level of the ribs, normally in proximity to hypaxial muscles, using antibodies recognizing GFP and collagen2a (Col2a) which marks the condensing cartilage of the ribs. In the double mutant, GFP⁺ cells are now located within and in the immediate vicinity of the rib.

Cell proliferation, another factor that may affect the capacity of Pax3⁺Pax7⁺ progenitor cells to contribute to muscle growth, does not appear to be affected in the GFP⁺ population in Pax3^{GFP/GFP}; Pax7^{LacZ/LacZ} mutant embryos (Fig. 4p, q). Notably, GFP-positive cells are still present at E13.5, when there is a marked deficit in skeletal muscle. They are mainly MyoD[−] (Fig. 4r, s, lower panels), and the remaining MyoD⁺ cells (3.7% of GFP-expressing cells, compared with 34.6% in the presence of Pax3 and/or Pax7, Fig. 4t) are all co-expressing terminal differentiation markers (Fig. 4r, s, lower panels, u). They probably correspond to differentiated cells dating from the first phase of embryonic myogenesis, when the myotome forms in the absence of Pax3 and Pax7 (Supplementary Fig. S3). This indicates that in the absence of both Pax proteins, myogenic progenitor cells at later embryonic and fetal stages are not specified as skeletal muscle. Instead, they downregulate transcription of the Pax genes, as shown in Fig. 4v, w, lower panels, where β-gal from the Pax7^{LacZ} allele is no longer detectable (3.2% versus 37.7%) in cells in which the more stable GFP reporter produced from the Pax3^{GFP} allele (Fig. 4s, Fig. 2s and Supplementary Fig. S2) is still present. Identical results to those shown for Pax7^{LacZ} were obtained with a Pax3^{IRESnLacZ} allele (data not shown). The GFP-positive mutant cells probably assume non-myogenic fates, as shown by their presence in bones, where they are associated with the expression of markers such as collagen 2a (Col2a, Fig. 4y), which is characteristic of cartilage cells.

Our results show that after the initial formation of the myotome, which has been a focus in the study of myogenic regulation^{2,16}, subsequent embryonic myogenesis depends on the expression of Pax3 and Pax7, making these factors key upstream regulators of the myogenic process. In the absence of both Pax proteins, resident muscle progenitor cells do not enter the myogenic programme. Double mutants die at mid-gestation, but given the persistence of this cell population and its demonstrated contribution to fetal muscles, we conclude that the skeletal musculature of the fetus also depends on Pax3 and Pax7. The apparent acquisition of satellite cell properties by the Pax3⁺Pax7⁺ cells in late fetal muscle suggests that the progenitor cells of postnatal and adult muscle also derive from this population. These cells are compromised in the Pax7 mutant¹⁰, but some satellite cells are still detectable, suggesting that they are specified correctly¹¹; furthermore, there is no deficit in satellite cells immediately postnatally (E.R., D. Montarras and M.B., unpublished data). This is consistent with a requirement for either Pax3 or Pax7 to generate the cell pool of muscle progenitors from which satellite cells derive. Complementary observations on the origin of the Pax3⁺Pax7⁺ population from the dermomyotome of the chick embryo using long-term lineage tracing²³ suggest that the progenitor cells of fetal and postnatal muscle derive from the paraxial mesoderm of the embryonic somite.

The resident muscle progenitor cells that we have identified constitute the principal proliferative cell population of developing skeletal muscle. It is important to determine whether these cells self-renew as a true stem cell population (see Figs 1m–p and 2g–i). Pax3 and Pax7 have an important role in conferring myogenic potential on these progenitor cells, thus assuring the major phases of skeletal muscle formation as the organism develops. Such cells represent a potentially exploitable resource in the context of cell therapy for muscle diseases.

METHODS

Mice. Generation and genotyping of the Pax3^{nLacZ/+}, Pax3^{IRESnLacZ/+} and Pax7^{LacZ/+} alleles have been previously described^{9,18,20}. The Pax3^{GFP/+} allele will be described elsewhere in detail. Briefly, enhanced (E)GFP replaces the Pax3 coding sequence of exon 1, as previously reported for DsRed in the Pax3^{DsRed/+} allele⁹.

X-Gal staining, histology, immunohistochemistry and *in situ* hybridization. X-Gal staining, histology, immunohistochemistry and whole-mount *in situ* hybridization were performed as previously reported⁹. Antibodies used were as follows: MyoD, either a rabbit polyclonal (Santa Cruz; 1:200 dilution) or a

mouse monoclonal antibody (clone 5.8A, DAKO; 1:200); desmin, mouse monoclonal antibody (clone D33, DAKO; 1:200); laminin, either a mouse monoclonal (clone 4C7, DAKO; 1:100) or a rabbit polyclonal antibody (Sigma; 1:200); Pax7, mouse monoclonal antibody (Developmental Studies Hybridoma Bank; 1:100); Pax3, mouse monoclonal antibody (provided by M. Bronner-Fraser; 1:100); β-gal, either a rabbit polyclonal (provided by J.-F. Nicolas; 1:500) or a mouse monoclonal antibody (clone Gal13, SIGMA; 1:100); phospho-histone H3, mouse monoclonal antibody (Cell Signalling; 1:200); TCF4, chicken polyclonal antibody (provided by G.R. Dressler; 1:200); MyHC, rabbit polyclonal antibody (provided by G. Cossu; 1:250); GFP, mouse monoclonal antibody (Biovalley; 1:1,000); Col2a, mouse monoclonal antibody (abcam; 1:100); Ki67, rabbit polyclonal antibody (Pharmingen; 1:200); cyclin A, rabbit polyclonal antibody (provided by A. Fernandez; 1:200).

Secondary antibodies were coupled to a fluorochrome, Alexa 350, 488, 546, 594 or 647 (Molecular Probes), used at 1:250 (Alexa 350 and 488) or 1:1,500 (Alexa 546, 594 or 647) dilutions. Images are obtained with Apotome Zeiss and Axiovision software. This system provides an optical section view reconstructed from fluorescent samples, using a series of 'grid projection' (or 'structured illumination') acquisitions. Figures were assembled using the Photoshop CS application (Adobe) and a PowerMacG4. Percentage figures given in the text on antibody labelling were based on the analysis of ≥6 sections from at least two embryos.

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LETTERS

A common somitic origin for embryonic muscle progenitors and satellite cells

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In the embryo and in the adult, skeletal muscle growth is dependent on the proliferation and the differentiation of muscle progenitors present within muscle masses. Despite the importance of these progenitors, their embryonic origin is unclear^{1,2}. Here we use electroporation of green fluorescent protein in chick somites³, video confocal microscopy analysis of cell movements, and quail–chick grafting experiments to show that the dorsal compartment of the somite, the dermomyotome, is the origin of a population of muscle progenitors that contribute to the growth of trunk muscles during embryonic and fetal life. Furthermore, long-term lineage analyses indicate that satellite cells, which are known progenitors of adult skeletal muscles⁴, derive from the same dermomyotome cell population. We conclude that embryonic muscle progenitors and satellite cells share a common origin that can be traced back to the dermomyotome.

During early somite differentiation, muscle growth in the trunk is entirely dependent on the generation of post-mitotic myocytes emanating from the four borders of the epithelial dermomyotome to form the primary myotome⁵. However, the dermomyotome is a transient structure that progressively disappears during development^{6,7} (Fig. 1a–c); this type of muscle formation therefore cannot account for the continuous and intense growth of muscles observed during late embryonic and fetal life, long after the dermomyotome has disappeared. Muscle progenitors, giving rise to muscles in culture and *in vivo*, and expressing early skeletal muscle markers, have been identified in all skeletal muscles of amniote embryos⁸. However, the timing and the developmental process through which they appear within the muscle masses are unknown.

In the adult, muscle growth and repair rely on the proliferation and the differentiation of satellite cells, located under the basal lamina of muscle fibres and recognized by their expression of specific molecular markers (such as the paired box transcription factor Pax7) (ref. 9). The embryonic origin of these satellite cells is unclear. Early experiments using the quail–chick chimera technique suggested a somitic origin¹⁰; however, this work did not identify the progenitor cell types at the origin of satellite cells or the developmental route that they followed to differentiate into satellite cells. More recently, alternative origins have been proposed for satellite cells, including cells derived from blood vessel walls (such as the aorta) and bone marrow cells, thus supporting a model where satellite cell development could take place independently of somitic myogenesis^{9,11–13}.

To identify the origin of embryonic muscle progenitors and satellite cells, we traced the fate of dermomyotome cells from the early stages of somite differentiation through to hatching. The early dermomyotome has an epithelial morphology; as somites mature, it undergoes a progressive epithelial–mesenchymal transition (EMT)¹⁴, which is characterized by the loss and/or the relocalization of the epithelial markers N-cadherin, β -catenin and F-actin at the adherens junctions located at the apical end of dermomyotomal

cells (Fig. 1a–l). The EMT of the dermomyotome is thought to be the first step in dermis formation¹⁵, and precedes the dorsal-ward migration of dermis progenitor cells from the somite towards the ectoderm.

Concomitant with the EMT, we observed a dramatic increase in the number of BrdU-positive cells within the myotome (Fig. 1m–p), suggesting that the EMT of the dermomyotome triggers the emergence of a proliferative cell population in the myotome, probably emanating from the dermomyotome. To verify this, we used electroporation of green fluorescent protein (GFP) to follow the movements of cells originating from the central region of the dermomyotome (Fig. 2a). Thirty-six hours after electroporation, as the EMT of the dermomyotome is initiated, a few GFP-positive cells were found within the myotome (Fig. 2b). As the incubation time increased, we observed a massive entry of GFP-positive cells emanating from the dermomyotome (Fig. 2c, e). This process was not uniform along the medio-lateral axis of the somite: in the lateral domain of the dermomyotome, all GFP-positive cells readily entered the myotome (L in Fig. 2c). In contrast, in the medial domain (M in Fig. 2c), most GFP-labelled cells remained dorsal to the primary myotome. However, by embryonic day (E)7.5 (120 h after electroporation) the medial domain was intensely GFP-labelled (Fig. 2f). This could be due to late entry of GFP-labelled cells from the dorso-medial dermomyotome or to intense proliferation of the few GFP-positive cells observed in this region before E7.5. When small regions of the dermomyotome were electroporated, the relative positions of GFP-positive cells within the myotome corresponded roughly to their original positions within the dermomyotome, indicating that little migration of dermomyotome-derived cells occurred within the plane of the myotome (not shown). This confirms previous data obtained using the quail–chick grafting technique^{15,16}. Many GFP-positive cells never entered the primary myotome, but remained between this structure and the ectoderm, presumably giving rise later to the development of dorsal dermis¹⁵ (arrowheads in Fig. 2d–f).

To determine the route that dermomyotomal cells take to enter the primary myotome, we used an *ex vivo* embryo-slice culture system combined with confocal time-lapse videomicroscopy. In contrast to what had been observed during primary myotome formation⁵, dermomyotomal cells did not transit across the dermomyotome borders; rather, they translocated directly from the central dermomyotome into the myotome (Fig. 2g–i). Real-time observation of this process (see Supplementary Video) revealed several additional features. First, a cell division preceded the translocation (Fig. 2h). Second, the plane of division was perpendicular to the apico-basal axis of the mother cell. Finally, the cytokinesis process was very rapid (6–12 min) and resulted in the translocation of one of the daughter cells into the myotome, while the other daughter remained in the dermomyotome (Fig. 2i).

We analysed the molecular characteristics of this population of

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cells as they entered the myotome, as well as their fate during embryonic development. Twelve hours after initiation of the dermo-myotome EMT (that is, 48 h after somite formation), 90% of GFP-positive cells present in the primary myotome expressed the early muscle progenitor cell marker Pax7. These GFP-positive cells were actively dividing (as shown by BrdU immunoreactivity) and only a

small percentage (10%) expressed the differentiated muscle marker myosin heavy chain (MyHC) (Fig. 3b and Supplementary Fig. 1a, d). As development proceeded, we observed a continuous increase in the contribution of the GFP-positive cell population to the differentiated muscle masses (shown by a gradual increase in MyHC/GFP double staining), while a portion of the GFP-positive cells (25% at E7.5)

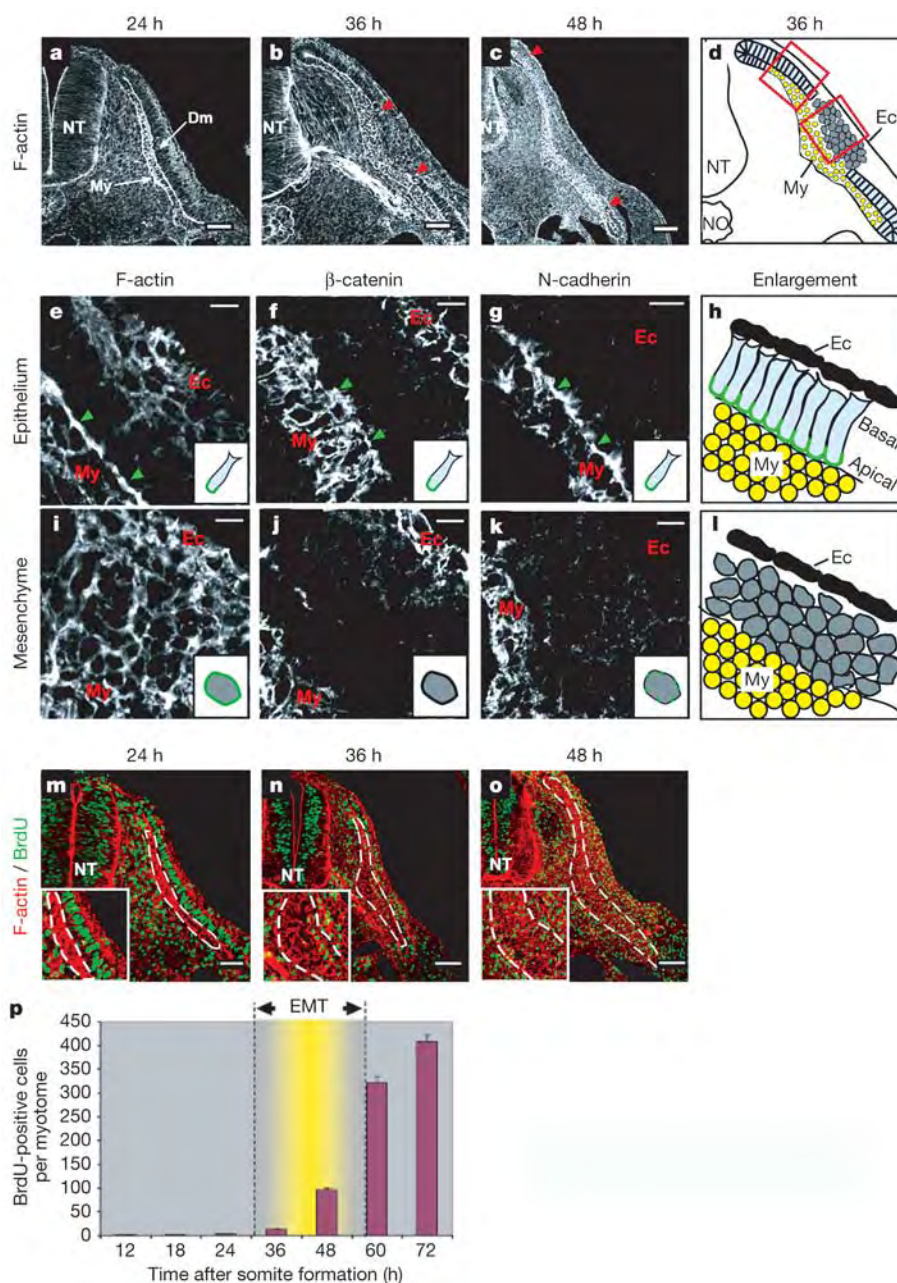


Figure 1 | The EMT of the dermomyotome triggers the emergence of a BrdU-positive cell population in the myotome. **a–c**, Confocal optical slices of somites (somite number 23), stained for F-actin 24 h (**a**), 36 h (**b**) or 48 h (**c**) after somite formation. NT, neural tube. **a**, At 24 h, the dermomyotome (Dm) is epithelial. **b**, **c**, Between 24 and 36 h, the central region of the dermomyotome begins a de-epithelialization process (red arrowheads in **b** and **c**) that progresses medially and laterally as development proceeds. **e–g** and **i–k**, Enlargements of sections through the epithelial (**e–g**) and the mesenchymal (**i–k**) regions of a dermomyotome, 36 h after somite formation (as shown in **b** and **d**). The regions shown in **e–g** and **i–k** correspond to the boxed areas in **d**, enlarged in **h** and **l**. Epithelial cells express F-actin (**e**, see also inset in Fig. 2a), β -catenin (**f**), and N-cadherin (**g**) at their apical end (green arrowheads and insets in **e–g**). The myotome (My)

expresses all three molecules, and the ectoderm (Ec) stains for F-actin and β -catenin. During EMT, the apical staining of all three markers is lost. F-actin relocates evenly in the cells (**i**, inset), overall β -catenin staining is reduced (**j**, inset) and N-cadherin staining becomes punctated (**k**, inset). **m–o**, Representative sections of interlimb somites stained for F-actin (in red) and BrdU (in green) to distinguish the myotome (highlighted with dotted lines) before (**m**) and during (**n**, **o**) the EMT. The myotome is devoid of BrdU-positive cells before EMT (**m** and inset) but a sharp increase in BrdU staining occurs at the time of the EMT (**n**, **o** and insets). **p**, Cell count of BrdU-positive cells within the myotome of a somite 12–72 h after its formation. Cell counts were performed on 12–15 sections per time point (>12,000 BrdU-positive cells counted); error bars show standard deviation. Scale bars, 50 μ m (**a–c**, **m**, **n**), 10 μ m (**e–k**), 100 μ m (**o**).

maintained early muscle progenitor characteristics (that is, Pax7 expression) and BrdU immunoreactivity during embryonic development (Fig. 3b and Supplementary Fig. 1b, c, e, f). Together, these data demonstrate that the cell population emerging from the dermomyotome as it undergoes an EMT contains embryonic muscle progenitors.

We next determined whether all embryonic muscle progenitors present in the myotome derive from the central region of the dermomyotome. A limitation of the electroporation technique is that it does not result in transfection of all targeted cells (in the best cases, 50% of the cells are electroporated, data not shown). Thus, the answers obtained using electroporation are more qualitative than truly quantitative. In contrast, the quail–chick grafting technique is particularly suitable for addressing this question, because we can use a marker of quail cells (the quail cell perinuclear antigen or QCPN) to

follow a population of grafted quail cells within a chick host during embryonic development.

We replaced the central region of the dermomyotome of four consecutive chick somites with central dermomyotome from quails (Fig. 3c). After a 24-h reincubation, we verified that the central dermomyotome was of quail origin (recognized by a QCPN quail-specific antibody), but that the dermomyotome borders were of chick origin (Supplementary Fig. 1g, h). After 48 h, quail dermomyotome cells entered the QCPN-negative, chick-derived primary

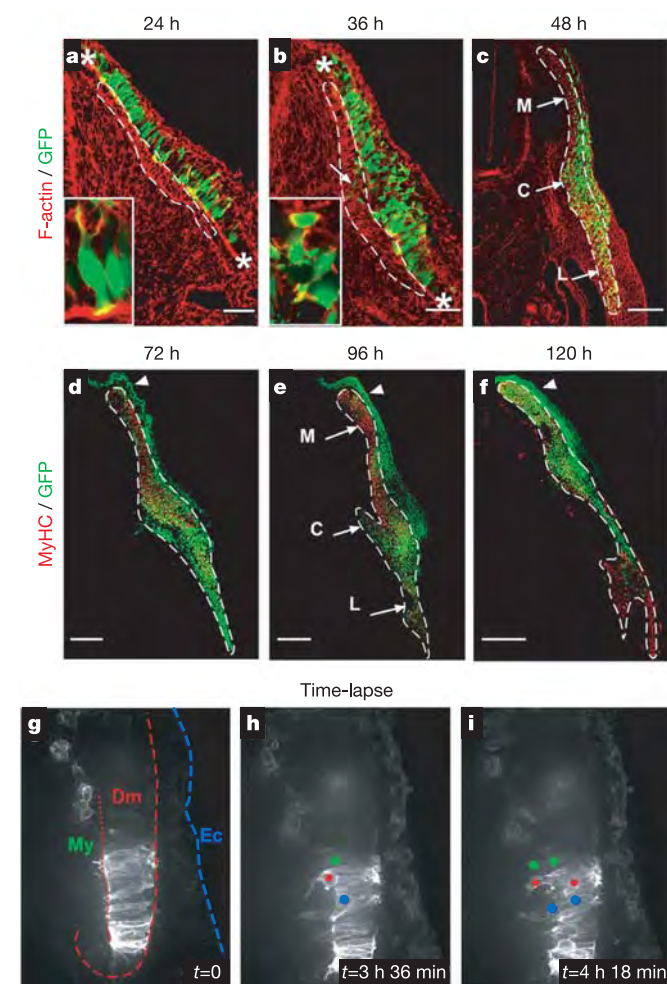


Figure 2 | Direct translocation of dermomyotome cells into the primary myotome. **a–f**, Sections of somites electroporated with GFP (medial and lateral borders indicated by white asterisks in **a**, **b**). **b**, 36 h after somite formation, a few GFP-positive cells (arrow) enter the primary myotome. **c–f**, After 48 h, many GFP-positive cells have entered the primary myotome. The dynamics of the distribution of GFP-positive cells is different in the lateral (L), central (C) and medial (M) domains. Insets in **a** and **b** show the morphological difference between epithelial (**a**) and mesenchymal (**b**) dermomyotome cells. **g–i**, Time-lapse confocal analysis (see Supplementary Video) showing the direct translocation of dermomyotome (Dm) cells into the myotome (My). Ec, ectoderm. 36 h after electroporation, somites were examined in an *ex vivo* embryo-slice culture system. Images in **h** and **i** show the cell division and translocation of three cells (coloured) from the dermomyotome into the myotome. Scale bars, 50 μ m (**a**, **b**) 200 μ m (**c**, **d**), 400 μ m (**e**), 500 μ m (**f**).

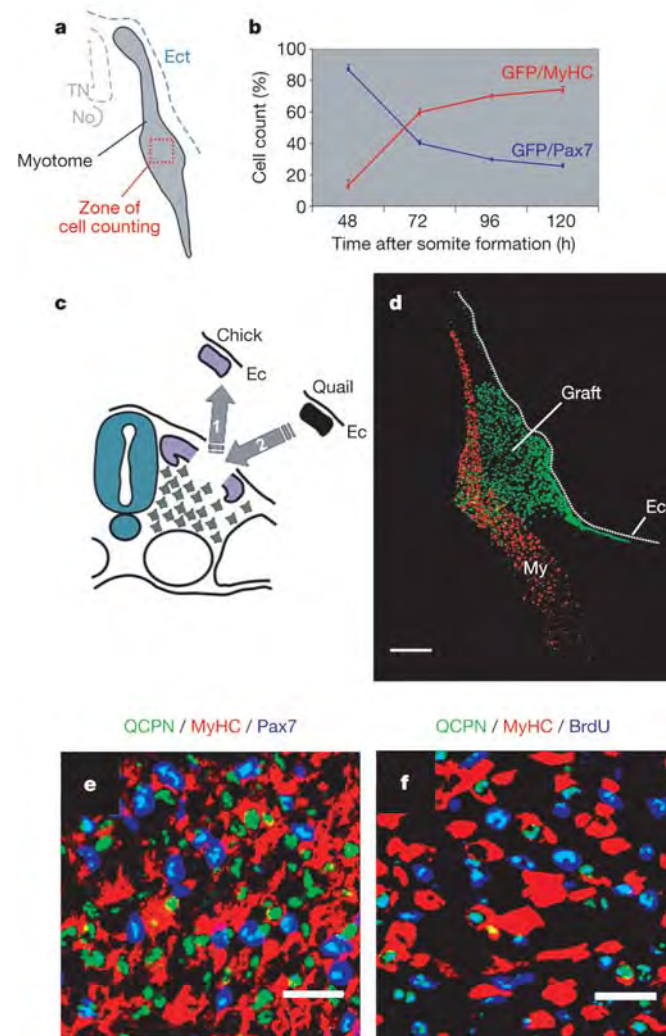


Figure 3 | Dermomyotome-derived cells contribute to embryonic muscle growth. Somites electroporated in the central dermomyotome were analysed 48–120 h after electroporation (data shown in Supplementary Fig. 2a–e). We counted the number of cells staining for either GFP + Pax7 or GFP + MyHC in a region corresponding to a central domain of the myotome (red dotted square in **a**). **b**, The contribution of the muscle progenitor population to muscle growth. Cell counts are expressed as a percentage of the GFP-positive cells in the entire myotome (counted zone delineated in **a**). For each time point, 12–15 sections were counted (>5,500 cells in total); error bars indicate standard deviation. Most GFP-positive cells expressed Pax7 48 h after electroporation. As development proceeds, a growing number of cells initiate MyHC expression. We did not observe any cells staining for both Pax7 and MyHC. At all stages, a small proportion of cells (about 5%) were neither Pax7- nor MyHC-positive. **c**, A schematic of the quail–chick grafting experiments. **d**, Section from a chimera analysed 72 h after grafting. In the region of the graft, numerous QCPN-positive quail cells (green) are seen within the myotome (red). **e**, **f**, Within the myotome of the embryo shown in **d**, most BrdU-positive (blue in **e**) or Pax7-positive (blue in **f**) cells express the QCPN antigen, observed as an irregular blue-green staining in the nuclei. Scale bars, 200 μ m (**d**), 10 μ m (**e**, **f**).

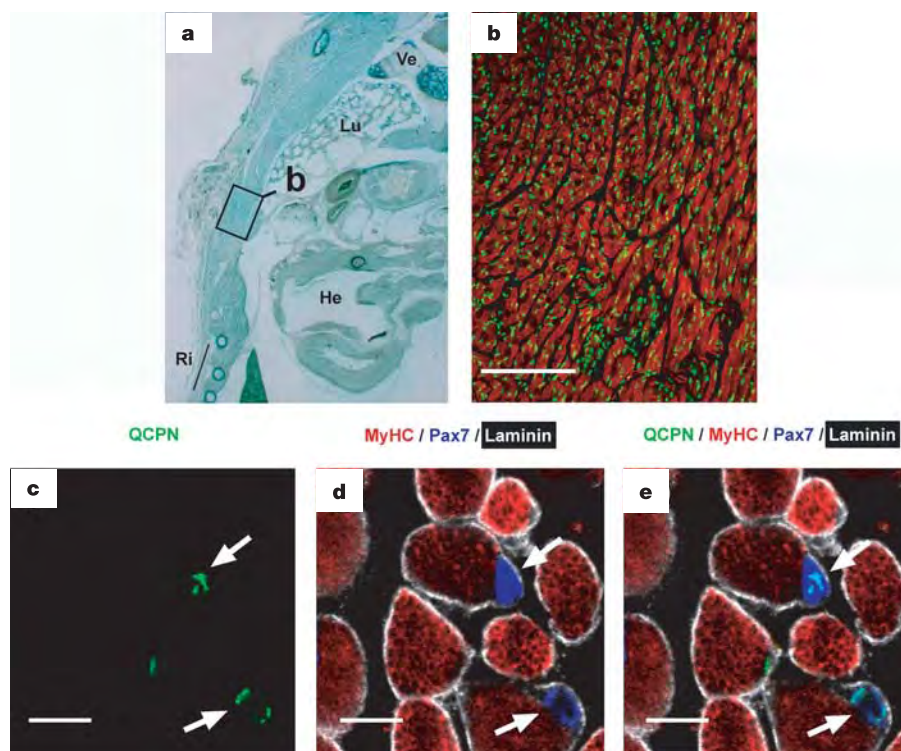


Figure 4 | Satellite cells are derived from the dermomyotome.

a, Histological view (light green staining) of a post-hatch quail-chick chimaera. Ve, vertebra; He, heart; Ri, rib; Lu, lung. **b**, Confocal view of the zone delineated in **a**, stained for MyHC (red) and the quail marker QPCN (green). The muscle is markedly colonized by quail cells. **c–e**, Confocal

images showing quadruple staining with antibodies against QPCN (green), MyHC (red), Pax7 (blue) and laminin (white). Quail (Pax7-positive) cells located under the basal lamina (arrows) identify satellite cells. Scale bars, 100 μ m (**b**), 5 μ m (**c–e**).

myotome (Supplementary Fig. 1i, j). After 72 h we observed, in the region of the graft, that the vast majority of Pax7-positive (97%) or BrdU-positive (93%) cells within the myotome stained for the quail marker (Fig. 3d–f). This suggests that most, if not all, proliferative embryonic muscle progenitors present in the myotome at that stage of development derive from the central dermomyotome. In a complementary experiment, we observed that the borders of the dermomyotome do not generate cells with characteristics of embryonic muscle progenitors, even after a long (7-day) reincubation period (Supplementary Fig. 2a–e), confirming that the contribution of the epithelial dermomyotome borders to the population of embryonic muscle progenitors is negligible.

We next determined whether a portion of dermomyotomal cells maintain myogenic potential into late fetal life and after hatching. In chick, it has been shown that cells with satellite cell characteristics appear within muscle masses during late fetal life, between E15 and E17 of development^{4,10,17,18}. At E18, satellite cells constitute the majority (about 85%) of the population of muscle progenitors, and at hatching (E21 in chick) they represent virtually all progenitors present in the muscle masses^{4,10,17,18}. Satellite cells are traditionally identified by their localization between the muscle fibre and the basal lamina¹⁹; however, Pax7 has recently been used as a reliable satellite cell marker. Using these two criteria, we determined whether we could identify, in late fetal life, satellite cells derived from the dermomyotome. Embryos were electroporated as described above and left to develop until late fetal life. In addition to GFP-labelled muscle fibres at E18, we found GFP-labelled, Pax7-positive cells tightly associated with muscle fibres and localized between the MyHC-positive muscle fibre and the laminin-positive basal lamina—a position that characterizes satellite cells (Supplementary Fig. 3a–d). As the GFP marker gradually becomes diluted with cell division, few GFP-positive satellite cells could be observed at E18. In

contrast, owing to the stability of the quail marker, we have been able to quantify the results obtained for the chimaeras. In the region of the graft, in E18 as well as in post-hatching chimaeras (Fig. 4a, d and Supplementary Fig. 3e–h), we found that 91% (E18) and 95% (post-hatch) of the satellite cells were of quail origin (cell count, $n = 600$ total in 4 specimens). A minor fraction of satellite cells could not be identified as quail, possibly owing to alternative origins of these cells or a limitation of the identification technique in quail cells (not all are recognized by the QPCN antibody, even in a bona fide quail; data not shown). Taken together, our data strongly suggest that most satellite cells present in skeletal muscles of the trunk derive from the central region of the dermomyotome.

In summary, our use of electroporation, *ex vivo* analyses of cell movements and the quail-chick grafting technique, has allowed us to analyse the morphogenesis and growth of skeletal muscles in birds. Our analyses have revealed that myogenesis follows two distinct stages. In the first stage of myotome morphogenesis, the epithelial borders of the dermomyotome generate postmitotic myocytes that readily organize into a primary myotome⁵. The second stage of myotome growth, described in the present work, is dependent on the emergence of a population of muscle progenitors within the primary myotome, triggered by the EMT of the dermomyotome. At a cellular level, the mode of cell translocation in the first and the second stages are clearly distinct. During the first stage of myotome morphogenesis, single cells translocate into the myotome while their neighbours remain epithelial. In the second stage, the epithelial integrity of the dermomyotome is disrupted before or concomitant with cell translocation. Notably, this change is correlated with the formation of dramatically different cell types (postmitotic myocytes versus proliferative muscle progenitors).

Finally, our approaches have allowed the characterization of the developmental route that leads to the emergence of satellite cells.

Previous studies had questioned the somitic origin of satellite cells by demonstrating that bone-marrow-derived cells can give rise, under specific experimental conditions, to satellite cells as well as muscle fibres. Recent work has shown that in adults, the satellite cell pool does not receive any significant haemopoietic input²⁰. Our data show that under normal developmental conditions, the vast majority, if not all, satellite cells are derived from the dermomyotome.

In conclusion, the demonstration of a common origin for embryonic muscle progenitors and satellite cells in amniotes opens new avenues for studying their roles in muscle growth and repair, and may lead to insights of therapeutic value.

METHODS

Electroporation. Newly formed epithelial somites were electroporated as previously described^{3,5}. In all cases, thoraco-lumbar (that is, interlimb) somites were electroporated. The positive and negative electrodes were positioned so that the dorsal region of somites (which gives rise to the dermomyotome) was electroporated. Embryos were screened by ultraviolet light examination one day after electroporation, and only embryos in which the central region of the dermomyotome was electroporated were kept for further analyses. A cytoplasmic form of GFP was used in all but the time-lapse experiment, for which a membranous form of GFP was used.

Antibodies, immunohistochemistry and confocal analysis. Phalloidin Alexa 456 (Molecular Probes) was used to detect F-actin. The following antibodies were used: mouse monoclonal antibodies against β -catenin (Transduction Laboratories), N-cadherin (C-8538, Sigma), BrdU (Becton-Dickinson), Pax7 (Hybridoma Bank), Light Meromyosin (MF20, Hybridoma Bank), GFP (Molecular Probes), QCPN (Hybridoma Bank); a rat monoclonal antibody against BrdU (Abcam); polyclonal antibodies against laminin (L-9393, Sigma) and GFP (Torrey Pines Biolabs). To analyse cell division, embryos were exposed to BrdU for 1 h. For simultaneous detection of 4 antigens by indirect fluorescence (Fig. 4d–f), spectral deconvolution was used. This was accomplished using a Zeiss confocal microscope (LSM 510 Meta). To identify satellite cells in pre-hatching chick embryos, 6 embryos electroporated at E3 were analysed at E18. In 4 of the 6 embryos, GFP-positive muscle fibres were found in the muscle masses. GFP-positive satellite cells were clearly identified in 3 out of the 4 embryos (see Fig. 4).

Time-lapse analysis. To observe the translocation movement of dermomyotome cells into the myotome, we used an *ex-vivo* embryo-slice culture system combined with confocal time-lapse videomicroscopy. Embryo slices (200–250- μ m thick) of the trunk region were placed in a 3-cm Petri dish in which the bottom had been replaced with a glass coverslip. Low-melting-point agarose (1%) in F-12 nutrient mixture (Ham) was poured around and above the embryo slice. The sample was placed in a heated chamber and examined on an inverted microscope (Axiovert, Zeiss) to which a Nipkow spinning disk confocal head (Ultraview, Perkin Elmer) was attached. Embryo slices can be kept in this culture system for at least 24 h. Metamorph image acquisition software was used to drive the microscope and the confocal head. Confocal stacks were taken at 6-min intervals.

Quail–chick grafting. We replaced the central region of the dermomyotome of 4 consecutive interlimb chick somites with central dermomyotome from quail. The surgeries were performed in differentiated somites (for example, somite stage IV–VII in a 30-somite chick embryo) with glass needles, taking care not to transplant the dermomyotome borders. The ectoderm was grafted together with the four pieces of dermomyotome attached to it. Embryos were analysed after 24 h ($n = 15$), 48 h ($n = 13$) and 72 h ($n = 10$). For long-term lineage analyses, we generated 84 quail–chick chimaeras, of which 13 reached the end of fetal life, 2 embryos were analysed at E18 and 2 chimaera were analysed after hatching.

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Notch/ γ -secretase inhibition turns proliferative cells in intestinal crypts and adenomas into goblet cells

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The self-renewing epithelium of the small intestine is ordered into stem/progenitor crypt compartments and differentiated villus compartments. Recent evidence indicates that the Wnt cascade is the dominant force in controlling cell fate along the crypt–villus axis¹. Here we show a rapid, massive conversion of proliferative crypt cells into post-mitotic goblet cells after conditional removal of the common Notch pathway transcription factor CSL/RBP-J (ref. 2). We obtained a similar phenotype by blocking the Notch cascade with a γ -secretase inhibitor. The inhibitor also induced goblet cell differentiation in adenomas in mice carrying a mutation of the *Apc* tumour suppressor gene. Thus, maintenance of undifferentiated, proliferative cells in crypts and adenomas requires the concerted activation of the Notch and Wnt cascades. Our data indicate that γ -secretase inhibitors, developed for Alzheimer's disease, might be of therapeutic benefit in colorectal neoplastic disease.

Notch genes encode large, single-transmembrane receptors that regulate a broad spectrum of cell fate decisions^{2,3}. Interaction between Notch receptors and ligands results in proteolytic cleavages of the receptor within the plane of the cell membrane. The resulting free Notch intracellular domain translocates into the nucleus, where it binds to the transcription factor RBP-J (CSL or CBF1), thus activating target gene transcription^{2,3}. The best-characterized Notch target genes are the hairy/enhancer of split (HES) and Achaete–Scute transcriptional repressors. These proteins in turn repress the expression of downstream genes^{4,5}.

A recent study describes increases in secretory cells at the cost of absorptive cells in the intestines of zebrafish that are mutant for *DeltaD* (a Notch ligand) and *mindbomb*⁶. Multiple Notch pathway components are expressed in murine crypts (M.E.v.G., unpublished data, and refs 7, 8). *Hes1* is a known Notch target gene in other tissues and is also expressed in crypts (Fig. 1a)^{9,10}. Animals deficient in *Hes1* die perinatally from severe neurological abnormalities. Analysis of the developing fetal intestine of *Hes1*^{-/-} mutant fetal mice revealed a relative increase in mucosecreting and enteroendocrine cells at the expense of absorptive enterocytes¹⁰. The crypt progenitor pool in the small intestine seemed unaffected, as judged by an analysis of proliferative activity. *Math1* is a target gene of Hes1-mediated repression in several organs, including the intestine^{11,12}. *Math1*^{-/-} mice die neonatally. Although the crypt–villus architecture was essentially undisturbed in the mutant mice, commitment towards the secretory lineage had entirely halted¹². These results have been interpreted to indicate that *Hes1* and *Math1* are required to skew

the fate of differentiating cells leaving the transit amplifying compartment towards an enterocyte or a secretory phenotype, respectively^{10–12}.

Indirect support for the control of intestinal cell fate by Notch stems from the use of γ -secretase inhibitors, as originally developed for Alzheimer's disease^{13,14}. Notch is one of several known γ -secretase substrates. Proteolytic processing of Notch by γ -secretase is an essential step after activation of the pathway. As a consequence, γ -secretase inhibitors block Notch pathway activation^{13,14}. Toxicological studies on rodents with these inhibitors have revealed increases in the size and number of mucosecreting goblet cells^{15–17}.

To directly assess the role of the Notch pathway in crypt homeostasis, we crossed mice carrying a floxed *Rbp-J* allele¹⁸ with transgenic mice (P450-Cre) carrying the Cre enzyme under the control of the inducible *Cyp1A* promoter¹⁹. After intraperitoneal injection of β -naphthoflavone, this *cre* allele is activated in several internal organs, including the epithelium of the small intestine and colon. At 60 h after *cre* induction by β -naphthoflavone injection in 3-month-old mice, standard histological analysis did not reveal marked morphological changes in the crypts (Supplementary Fig. 1a, b). *In situ* hybridization revealed a decrease in *Hes1* expression (not shown). In accordance with observations made in

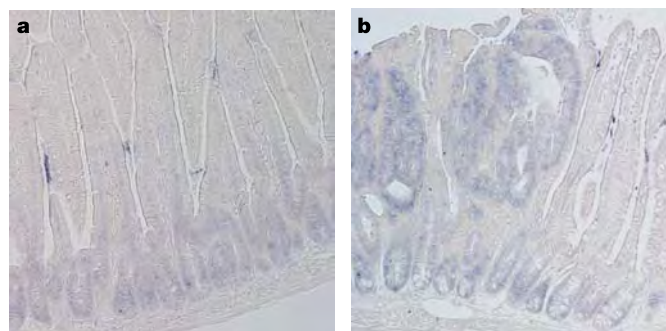


Figure 1 | Notch signalling pathway components are expressed in crypts of the small intestine. Expression of all Notch receptors and ligands in adult small intestine was analysed by *in situ* hybridization, essentially confirming refs 6 and 7 (M.E.v.G., unpublished data). **a, b**, Expression of the presumptive Notch target gene *Hes1* implies activity of the Notch cascade in the intestinal crypt (**a**) and in *Apc*^{Min} tumours (**b**). The sense control was negative (data not shown).

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Hes1 mutant mice, the *Math1* gene was derepressed. Nuclear Math1 protein, normally present only in secretory cells (Supplementary Fig. 1c), was abundant throughout the crypt compartment (Supplementary Fig. 1d; quantified in Supplementary Table 1). Moreover, there was a modest increase in goblet cell number as revealed by staining with periodic acid-Schiff (PAS) (compare Supplementary Fig. 1f with Supplementary Fig. 1e).

Histological analysis at day 5 after *cre* induction revealed a marked crypt phenotype. Phenotypic alterations occurred in more than 95% of all crypts of the small intestine and colon, underscoring the efficiency of the induction of the *cre* transgene by β -naphthoflavone. The following observations were made in the small intestine. Paneth cells still resided in near-normal numbers at the bottom of the crypts. However, the rapidly dividing transit amplifying compartment,

which normally occupies the remainder of the crypt, was entirely replaced by post-mitotic goblet cells, as identified by morphology under haematoxylin/eosin staining (HE) (compare Fig. 2b with Fig. 2a) and PAS staining (compare Fig. 2d with Fig. 2c). This was confirmed by staining for the Math1 protein (compare Fig. 2f with Fig. 2e; quantified in Supplementary Table 1), whereas *Hes1* gene expression had become undetectable (Supplementary Fig. 2a, b). Staining for Ki67 (compare Fig. 2h with Fig. 2g) and analysis of bromodeoxyuridine (BrdU) incorporation (quantified in Supplementary Table 1) revealed that essentially all epithelial cell division had halted. No significant increases in apoptosis were observed in crypts, as revealed by staining for caspase-3 (compare Supplementary Fig. 2d with Supplementary Fig. 2c). Numbers of enteroendocrine cells were unchanged, as revealed by staining for synaptophysin

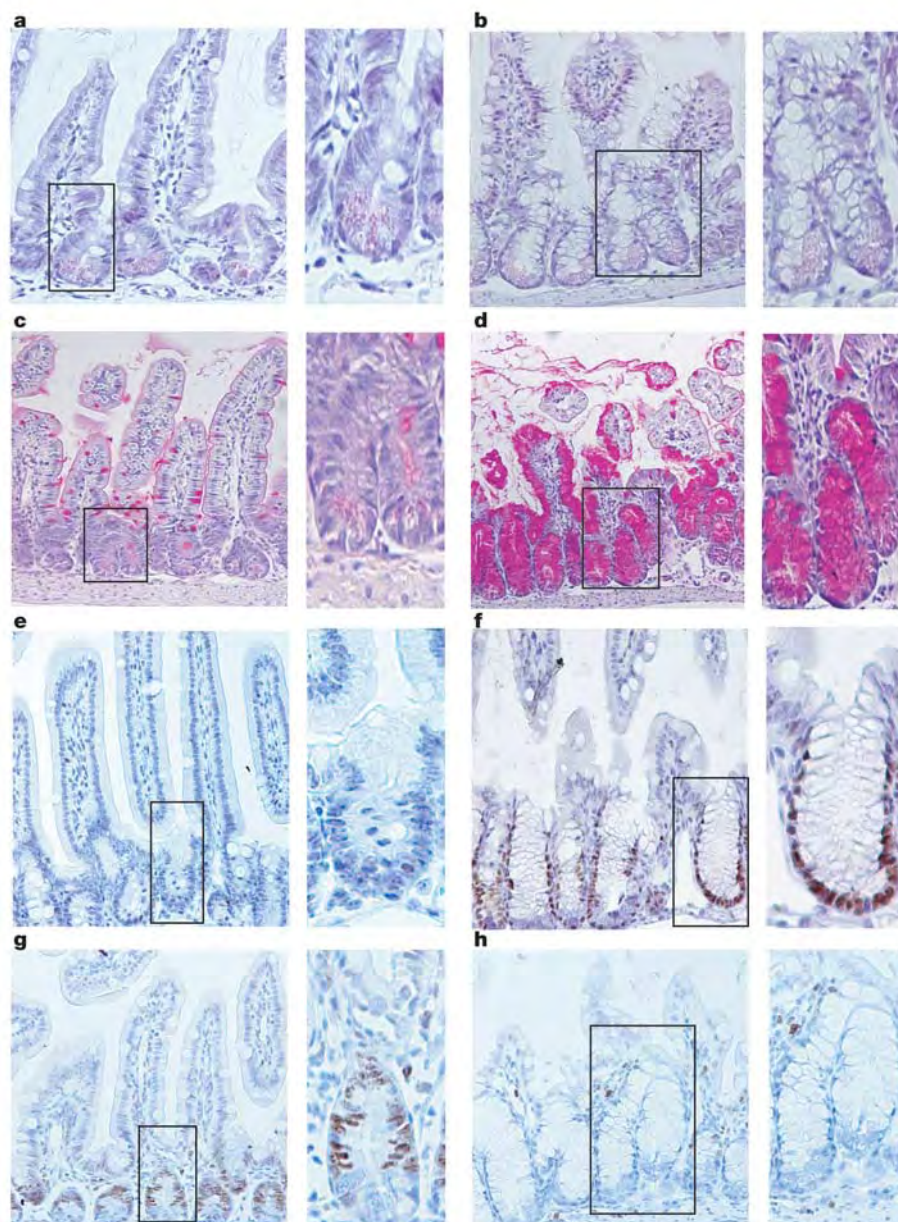


Figure 2 | Disruption of Notch signalling pathway induces goblet cell conversion of crypt proliferative cells. **a–h**, Immunohistochemical analysis of small intestine from RBP-J^{floxex/floxex}/P450-Cre mice (**b, d, f, h**) and control RBP-J^{floxex/floxex} mice (**a, c, e, g**). Mice were injected at days 0 and 2.5 with β -naphthoflavone. The analysis shows a complete replacement of the transit amplifying compartment by goblet cells as shown by

haematoxylin/eosin staining (HE) (**b** versus **a**) and PAS staining (**d** versus **c**). This notion was confirmed by staining for Math1 protein (**f** versus **e**). Staining for the proliferation marker Ki67 (**h** versus **g**) showed that essentially all epithelial cell division had halted. An enlargement of the boxed portion in the left-hand micrograph of each pair is shown in the right-hand micrograph.

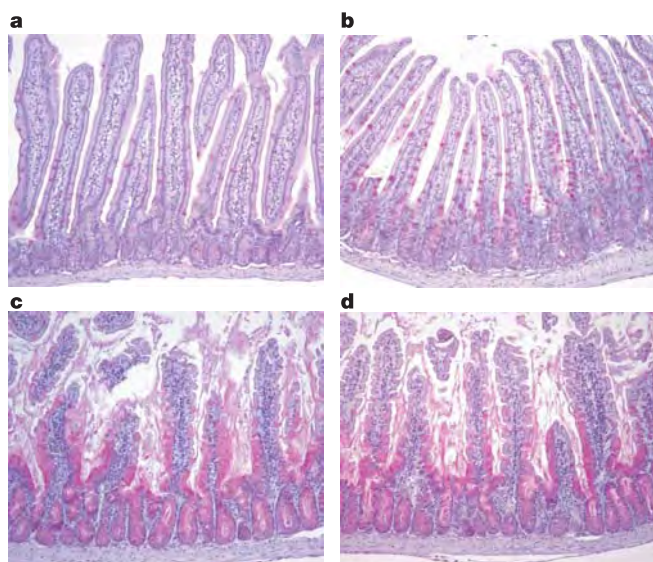


Figure 3 | Conversion of proliferative crypt cells into post-mitotic goblet cells by the γ -secretase inhibitor DBZ. C57BL/6 mice were injected intraperitoneally with $0 \mu\text{mol kg}^{-1}$ (a), $3 \mu\text{mol kg}^{-1}$ (b), $10 \mu\text{mol kg}^{-1}$ (c) and $30 \mu\text{mol kg}^{-1}$ (d) DBZ, daily for 5 days. At $3 \mu\text{mol kg}^{-1}$ DBZ, goblet cell numbers slightly increased as shown by PAS staining, whereas at 10 and $30 \mu\text{mol kg}^{-1}$ the conversion of proliferative crypt cells into post-mitotic goblet cells was complete.

(compare Supplementary Fig. 2f with Supplementary Fig. 2e). The Wnt signalling pathway remained active, as demonstrated by the presence of nuclear β -catenin (compare Supplementary Fig. 3h with Supplementary Fig. 3g). Essentially identical observations were made in the colon. Epithelial proliferation halted, whereas the numbers of goblet cells, already abundant in the colon, increased further (not shown).

We also inducibly inactivated the RBP-J gene by using a different Cre transgene, the tamoxifen-inducible villin-Cre-ERT2, which is expressed under the control of the villin promoter and is expressed exclusively in intestinal epithelium²⁰. Twelve-day-old RBP-J^{flox/flox}/villin-Cre-ERT2 mice and RBP-J^{flox/flox} littermate controls were injected intraperitoneally with tamoxifen on five consecutive days. The mice were analysed 6 and 12 days after the last injection. At both time points, histological analysis revealed goblet cell conversion. A nearly complete conversion of transit amplifying cells into post-mitotic goblet cells was observed 12 days after Cre induction (Supplementary Fig. 3a, b).

An explanation for the discrepancy between the observed phenotype in the RBP-J^{flox/flox}/P450-Cre mice and that of *Hes1* knockout mice might be the presence of other *Hes* genes. Indeed, we detected *Hes5* and *Hes6* expression in the crypts. Whereas *Hes6* expression seemed highest in the cells directly above the Paneth cell compartment, *Hes5* was expressed almost exclusively within the Paneth cell compartment (Supplementary Fig. 4a and b). *Hes6* expression was virtually abrogated after deletion of *Rbp-J*, whereas *Hes5* expression persisted in Paneth cells (Supplementary Fig. 4b and d, respectively).

As an alternative tool to block Notch signalling *in vivo*, we synthesized the γ -secretase inhibitor dibenzazepine (DBZ)¹⁶ to more than 99.9% purity. DBZ blocked Notch cleavage in a cell-based assay with a half-maximal inhibitory concentration of less than

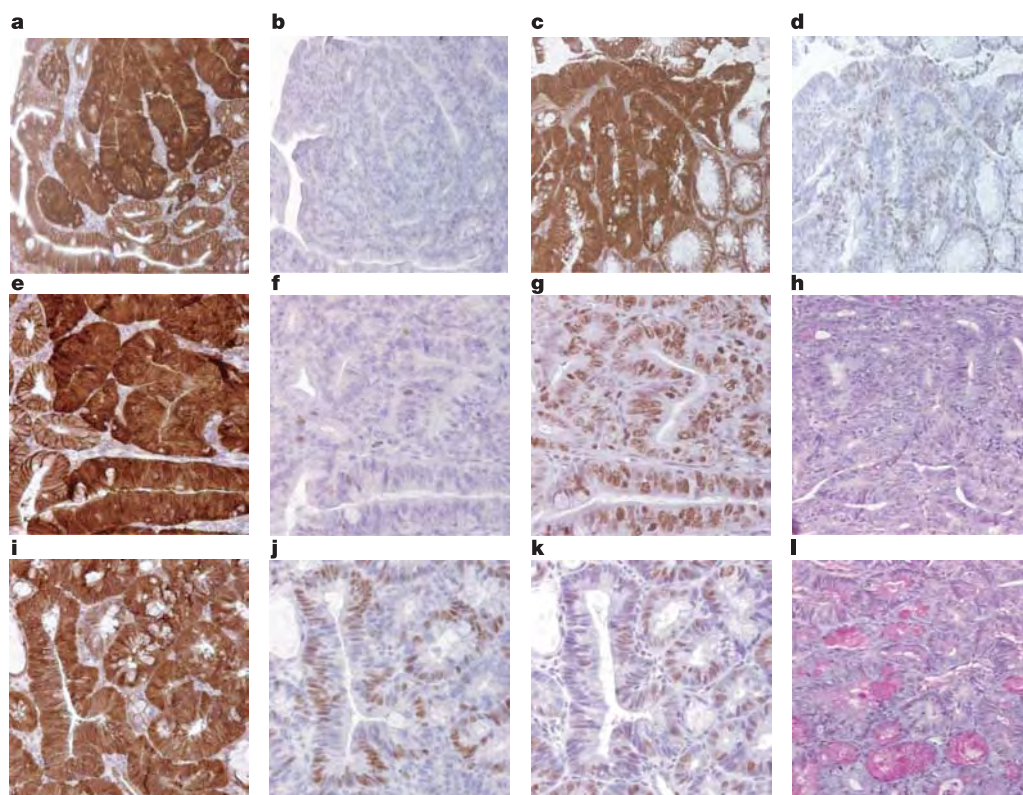


Figure 4 | Conversion of proliferative cells in the *Apc*^{Min} tumour into post-mitotic goblet cells by the γ -secretase inhibitor DBZ. a–l, *Apc*^{Min} mice were treated on alternate days without DBZ (a, b, e–h) or $10 \mu\text{mol kg}^{-1}$ DBZ (c, d, i–l) for 10 days, after which intestines were examined histologically by

serial sectioning. Staining for β -catenin delineated the adenomas (a, c, e, i). DBZ treatment induced Math1 (b versus d; at a higher magnification, f versus j), decreased Ki67 expression (k versus g) and induced PAS expression (h versus l).

2 nM (not shown). After the pharmacokinetic studies of the original study¹⁶, the compound was injected daily intraperitoneally at 0, 3, 10 and 30 $\mu\text{mol kg}^{-1}$ into C57BL/6 mice for 5 days. At 10 and 30 $\mu\text{mol kg}^{-1}$, goblet cell conversion was complete within 5 days of starting intraperitoneal injections, as shown by PAS staining (Fig. 3c, d, respectively). Moreover, cell proliferation had entirely halted and histological markers (Ki67 and Math1) revealed that the tissue changes were indistinguishable from those observed after the deletion of *Rbp-J* (results not shown). At 3 $\mu\text{mol kg}^{-1}$ DBZ, goblet cell numbers, as shown by PAS staining, increased slightly (Fig. 3b) in comparison with the intestine derived from the control mice (Fig. 3a).

We interpreted the combined genetic and pharmacological findings as indicating that Notch signalling is essential within the crypt compartment itself to maintain the undifferentiated state of the crypt progenitors. A complementary gain-of-function study yielded the reciprocal phenotype. Transgenic expression of the Notch intracellular domain in the intestinal epithelium results in a block of differentiation of secretory cells and an expansion of immature progenitor cells²¹.

Intestinal adenomas result from mutational activation of the Wnt pathway, most commonly due to the loss of the intestinal tumour suppressor gene *Apc* (reviewed in ref. 22). We have recently reported a remarkable symmetry between colorectal cancer cells and proliferative crypt progenitors in terms of the expression of a Wnt target gene programme²³. To investigate whether the symmetry between crypts and intestinal neoplasia extends to the Notch pathway, we studied the expression of various Notch pathway components and target genes in adenomas that spontaneously occur in multiple intestinal neoplasia (Min) mice, which carry a heterozygous mutation of the *Apc* gene (*Apc*^{Min})²⁴. In general, expression of receptors and ligands in adenomas closely followed expression in crypts (M.E.v.G., unpublished data). In addition, *Hes1* expression, indicative of active Notch signalling, not only occurred in crypts but was also observed uniformly in adenomas of all sizes in the intestines of *Apc*^{Min} mice (Fig. 1b). This observation implied that, as in crypts, the Notch and Wnt pathways are simultaneously active in proliferative adenoma cells.

We then asked whether Notch pathway activity was essential for the maintenance of the proliferative phenotype of adenoma cells. We elected to inhibit the Notch pathway pharmacologically through the use of the γ -secretase inhibitor DBZ¹⁶. We initiated the treatment of 8-week-old *Apc*^{Min} mice, which at this age carry 30–60 macroscopically detectable adenomas (polyps) in the small intestine and 0–3 adenomas in the colon. Two mice each were treated with 0, 3, 10 or 30 $\mu\text{mol kg}^{-1}$ DBZ for 10 days, after which intestines were examined histologically by serial sectioning. Staining for β -catenin delineated the adenomas, which were often embedded in an accumulation of hyperplastic yet untransformed normal crypts (Fig. 4a, c, e, i). DBZ at 10 or 30 $\mu\text{mol kg}^{-1}$ readily induced Math1⁺/PAS⁺/Ki67⁺ cells within adenomas (Fig. 4d, j–l versus 4b, f–h), whereas the effects at 3 $\mu\text{mol kg}^{-1}$ were minimal, as were the effects on normal crypts (not shown). Different conversion rates were observed in individual adenomas, even within the same animal. To quantify this, 100 adenomas from mice treated with 10 $\mu\text{mol kg}^{-1}$ DBZ were analysed by determining the percentage of Math1⁺ nuclei. In 8% of the adenomas more than 50% of all epithelial cells were converted into Math1⁺ cells. In 20% of the adenomas 10–50% conversion occurred; 28% showed 1–10% conversion, and 46% showed no conversion (examples are given in Fig. 4d, j). Goblet cell conversion was never observed in untreated *Apc*^{Min} mice: in each of 100 adenomas analysed, fewer than 1% Math1⁺ goblet cells were observed. The treatment intensity and duration with this particular compound were limited by the concurrent changes in the normal epithelium. Nevertheless, the observations showed that adenoma cells can be forced to differentiate upon inhibition of the Notch pathway.

Inhibition of the Wnt/ β -catenin/Tcf4 pathway induces a complete

loss of crypt epithelial progenitors²⁵. The combined observations indicate that multipotent crypt progenitors might be maintained only when both Notch and Wnt pathways are active. Specific inhibition of the Notch pathway drives the cells out of cycle towards a secretory fate, even while the Wnt cascade remains active. Conversely, inhibition of the Wnt cascade either by the deletion of *Tcf4* (ref. 25) or β -catenin¹⁹ or by the transgenic expression of the soluble Wnt inhibitor Dickkopf-1 (ref. 26) drives the cells towards an enterocyte fate. Taken together, the Notch and Wnt signalling cascades synergize as gatekeepers of self-renewal in the intestinal epithelium. A wealth of evidence has indicated that the Wnt cascade might be the major driving force behind the proliferative potential of adenomas and adenocarcinomas of the intestine. The present data indicate that active Notch signalling might be equally important in maintaining the undifferentiated state of *Apc*-mutant neoplastic cells. Activating mutations occur relatively frequently in the *Notch1* gene in T-cell leukaemias²⁷. It might be expected that such mutations occur also in colorectal cancer.

The Wnt cascade presents a rather unfavourable target for drug development, because the segment of the cascade downstream of the *Apc* tumour suppressor protein is driven entirely by protein–protein interactions²². The Notch pathway might provide an alternative targeted-drug strategy for the treatment of intestinal neoplastic diseases such as familial adenomatous polyposis or sporadic colorectal cancer. Multiple γ -secretase inhibitors of various chemical origins have been developed for the treatment of Alzheimer's disease^{13,14,28}. Increases in intestinal goblet cell numbers in animal toxicity studies^{15–17} have been noted as the principal unwanted side effect of these compounds. However, we suggest that the current experiments provide a proof of principle for the notion that these γ -secretase inhibitors could be developed into therapeutic modalities for colorectal neoplasia.

METHODS

Generation of RBP-J^{floxex}/floxex/P450-Cre and RBP-J^{floxex}/floxex/vil-Cre-ERT2 mice. The transgenic line Ahcre/P450-Cre (ref. 19) was crossed with floxed RBP-J mice¹⁸ to generate RBP-J^{floxex}/floxex/P450-Cre mice. The Cre enzyme was induced at 3 months of age by a single intraperitoneal injection at day 0, or intraperitoneal injections at days 0 and 2.5, of 200 μl β -naphthoflavone (10 mg ml⁻¹; Sigma Aldrich) dissolved in corn oil. The mice were killed on days 2.5 and 5 respectively. The transgenic line vil-Cre-ERT2 (ref. 20), in which Cre expression is specifically expressed in the intestine, was crossed with the floxed RBP-J mice¹⁸ to generate homozygous floxed RBP-J/vil-Cre-ERT2 mice, as well as various genotypic controls. After tamoxifen injection the Cre enzyme becomes active. Tamoxifen was prepared as described²⁰. Mice were injected intraperitoneally with tamoxifen (1 mg per 10 mg body weight).

Tissue sample preparation, immunohistochemistry and *in situ* hybridization. The intestinal tract was flushed gently with cold PBS followed by a flush with formalin. The small intestine was fixed in formalin at 21 °C for 16 h. The tissues were sectioned (2–6- μm slices). After dewaxing and hydration, sections were pretreated with peroxidase blocking buffer (120 mM Na₂HPO₄, 43 mM citric acid, 30 mM Na₂SO₄, 0.2% H₂O₂, pH 5.8) for 15 min at room temperature. Antigen retrieval was performed by boiling samples for 20 min in 10 mM sodium citrate buffer pH 6.0. Antibodies used were mouse anti-Ki67 (1:100 dilution; Novocastra), mouse anti- β -catenin (1:50 dilution; Transduction Labs), mouse anti-bromodeoxyuridine (1:500 dilution; Becton Dickinson), rabbit anti-Math1 (1:50 dilution), rabbit anti-synaptophysin (1:200 dilution; Dako) and rabbit anti-caspase-3 (1:300 dilution; Cell Signalling). Incubation of antibodies was performed overnight in BSA in PBS at 4 °C for antibodies directed against caspase-3 and Math1, and for 1 h at room temperature for antibodies directed against Ki67, bromodeoxyuridine, synaptophysin and β -catenin. In all cases, the Envision⁺ kit (Dako) was used as a secondary reagent. Stainings were developed with DAB. Slides were counterstained with haematoxylin and mounted. *In situ* hybridizations were performed as described²⁹. The probes used for *in situ* hybridization were as described⁷.

Treatment of animals with the γ -secretase inhibitor DBZ. DBZ¹⁶ (3 g) was custom-synthesized to more than 99.9% purity by Syncom. DBZ was suspended finely in 0.5% (w/v) hydroxypropylmethylcellulose (Methocel E4M) and 0.1% (w/v) Tween 80 in water. Injections were performed intraperitoneally for the indicated periods with the indicated amounts.

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LETTERS

Notch signals control the fate of immature progenitor cells in the intestine

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& Spyros Artavanis-Tsakonas^{1,4}

The Notch signalling pathway plays a crucial role in specifying cellular fates in metazoan development by regulating communication between adjacent cells^{1,2}. Correlative studies suggested an involvement of Notch in intestinal development. Here, by modulating Notch activity in the mouse intestine, we directly implicate Notch signals in intestinal cell lineage specification. We also show that Notch activation is capable of amplifying the intestinal progenitor pool while inhibiting cell differentiation. We conclude that Notch activity is required for the maintenance of proliferating crypt cells in the intestinal epithelium.

The Notch transmembrane receptor is the central element of a signalling pathway that controls a broad spectrum of metazoan cell fates and developmental processes through local cell interactions^{1,2}. It is now well established that signals through the Notch receptor are involved in the development of several cell types and that the modulation of these signals can markedly affect differentiation, proliferation and apoptotic events^{3,4}. Activation of the pathway has been shown to be a potent inhibitor of differentiation in different developmental contexts and has been associated with the amplification of some somatic stem cells, such as the neural and haematopoietic stem cells^{5,6}.

The intestinal epithelium is a model of tissue renewal from a source of multipotent stem cells. Throughout adulthood, the proliferation, cell fate specification and differentiation of intestinal cells correlate with migration along the crypt–villus axis. The rate of cell turnover in the gastrointestinal tract is remarkably rapid, with new precursor cells being constantly generated in the crypts of Lieberkühn. Once they become differentiated, these cells migrate upwards towards the apex of the villi and are eventually shed into the gut lumen.

The mechanisms that control the differentiation of epithelial cells in the intestine remain largely unknown. Nonetheless, the analysis of mice deficient for the basic helix–loop–helix proteins *Hes-1*, *Math-1* and *neurogenin-3*, all of which are transcriptional targets of Notch signalling in other tissues^{7–9}, have indirectly implicated the Notch pathway in the regulation of the earliest intestinal cell fate decisions. If these transcription factors are indeed controlled by Notch in this tissue, we reasoned that the activation of the Notch receptor should affect their expression. In addition, such an approach would allow us to evaluate the role of Notch signal activation on the differentiation and proliferation of intestinal precursors. We therefore used the villin promoter to target the expression of a constitutively active form of the mouse Notch 1 receptor (*N1ic*) in all cells of the intestinal epithelium, including the stem cells of the crypts^{10–14}.

Doubly transgenic mice carrying both *Villin-Cre*¹⁴ and *Rosa-Notch*¹⁵ (*Rosa-Notch/Cre*⁺) transgenes (see Supplementary Fig. S1) are born at mendelian ratios, but they die within 3 days of birth

(*n* = 57). At birth, *Rosa-Notch/Cre*⁺ mice are macroscopically indistinguishable from the *Rosa-Notch/Cre*[−] littermates. Within 24 h, however, the *Rosa-Notch/Cre*⁺ pups become runted, show signs of malnutrition and display a markedly altered architecture of the intestinal epithelium accompanied by an increase in apoptosis

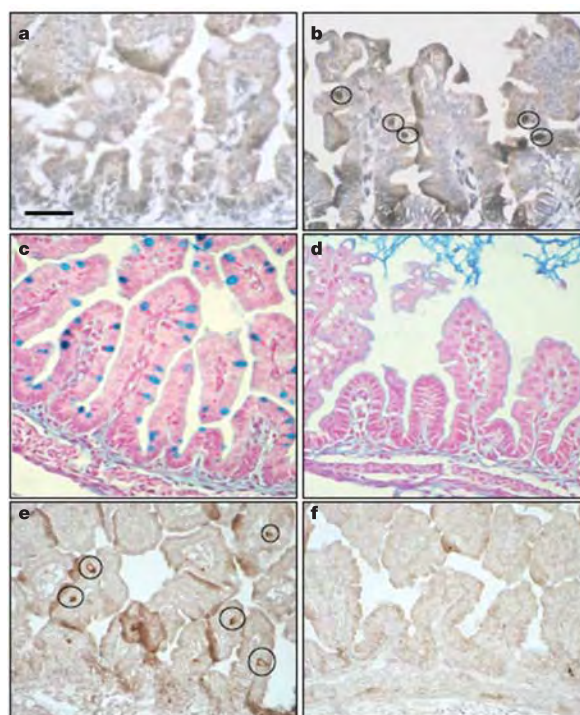


Figure 1 | Activation of Notch signalling induces apoptosis and impairs goblet and enteroendocrine cell differentiation. **a, b**, Paraffin sections of P0 *Rosa-Notch/Cre*[−] (**a**) and *Rosa-Notch/Cre*⁺ (**b**) mice were stained with an antibody that recognizes cleaved caspase-3. In villi from the proximal duodenum of *Rosa-Notch/Cre*⁺ mice several apoptotic figures can be seen (circled in **b**), whereas *Rosa-Notch/Cre*[−] neonatal intestines do not show apoptotic figures at this developmental stage (**a**). **c, d**, Alcian blue staining reveals several goblet cells in *Rosa-Notch/Cre*[−] duodenum (stained blue in **c**) but not in the *Rosa-Notch/Cre*⁺ P0 mice (**d**). **e, f**, Representative sections of P0 *Rosa-Notch/Cre*[−] (**e**) and *Rosa-Notch/Cre*⁺ (**f**) mice stained with the pan-endocrine marker chromogranin A/B. Several chromogranin-positive cells are evident in the small intestine of *Rosa-Notch/Cre*[−] mice (circled in **e**), but not in the *Rosa-Notch/Cre*⁺ mice (**f**). Scale bar, 80 μ m.

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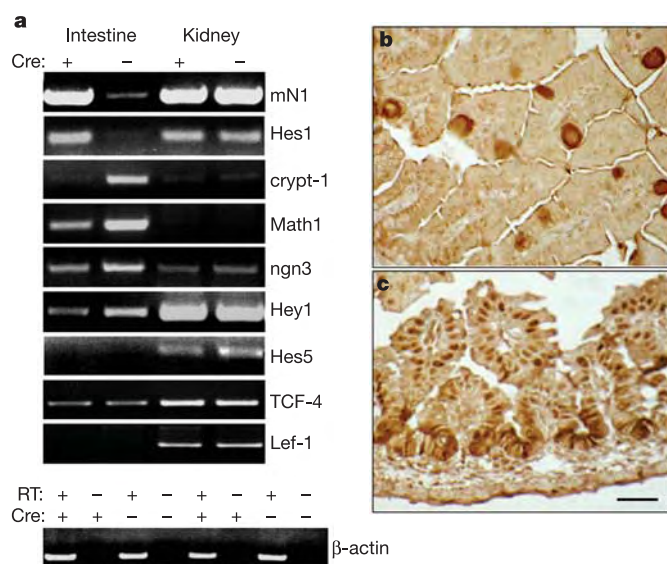


Figure 2 | Notch activation upregulates Hes-1 and represses the transcription of Math1 and ngn3. **a**, RT-PCR from P0 intestines shows elevated expression of N1ic and Hes-1 in Rosa-Notch/Cre⁺ intestines, compared with the Rosa-Notch/Cre⁻ controls. Consequently, the levels of Math1 and ngn3 are repressed. Notch activation represses the Paneth cell differentiation marker cryptdin-1, whereas the intestinal levels of Hes-5 and Hey-1 mRNA are not significantly affected. The expression of the Wnt signalling target genes TCF-4 and LEF-1 is not affected by Notch activation. RNA extracted from the kidneys of the same mice was used to assess specificity of expression for the intestine. Bottom, β -actin mRNA level was used as a control. **b**, **c**, Immunohistochemical analysis of Hes-1 expression in P0 intestines from Rosa-Notch/Cre⁻ (**b**) and Rosa-Notch/Cre⁺ (**c**) mice reveals strong staining for Hes-1 (dark brown) in the nuclei of all cells in the intestinal epithelium of N1ic-expressing mice (**c**). Note that the goblet cells in Rosa-Notch/Cre⁻ intestine show non-specific staining with this antibody (**b**). Scale bar, 80 μ m.

(Fig. 1a, b). Using secretory cell differentiation markers, we find that neonatal intestines from postnatal day 0 (P0) Rosa-Notch/Cre⁺ mice have a complete depletion of mucosecreting goblet cells in all intestinal tracts (Fig. 1c, d). In addition, we notice a marked reduction in entero-endocrine cells, as judged by the lack of staining with the pan-endocrine marker chromogranin¹⁶ (Fig. 1e, f) or with Grimelius silver stain (data not shown). Last, the low expression of cryptidin-1, as detected by reverse transcriptase polymerase chain reaction (RT-PCR), indicates that the differentiation of the Paneth cells¹⁷ in the Rosa-Notch/Cre⁺ intestines is also compromised (Fig. 2a). These observations indicate that the villin promoter might drive the expression of activated Notch in early progenitor cells and consequently that the differentiation of all secretory cell lineages is inhibited.

Microscopic examination of earlier developmental stages revealed that already at embryonic day 18.5 (E18.5), N1ic expression affects the architecture of the villi, the differentiation of secretory cell lineages along the duodenal-ileal axis and the cranial-to-caudal wave of intestinal differentiation (see Supplementary Fig. S2). As normal antero-posterior development proceeds, the absorptive surface of the small intestine is markedly increased by the presence of numerous villi. The large intestine, in contrast, lacks villi but is characterized by the occurrence of several crypts of Lieberkühn, which develop as invaginations into the intestinal mucosa (Supplementary Fig. S2c). The proximal intestinal tracts of Rosa-Notch/Cre⁺ embryos have a decreased number of villi (Supplementary Fig. S2b), whereas the distal tracts, corresponding to the large intestine in a wild-type animal, show several fingerlike disorganized villous structures and seem clearly different from the normal flat colonic epithelium (Supplementary Fig. S2d).

Transcriptional analysis revealed a direct correlation between N1ic expression and elevated levels of Hes-1 transcription in the intestinal epithelium of Rosa-Notch/Cre⁺ mice (Fig. 2a). The upregulation of Hes-1 is also reflected by the high concentrations of nuclear Hes-1 protein seen in all cells lining the intestinal lumen (Fig. 2b, c). In contrast, N1ic activation in the intestine does not influence the expression of either Hes-5 or Hey-1, two additional HLH transcription factor genes that are targeted by Notch signals in other developmental contexts¹⁸ (Fig. 2a).

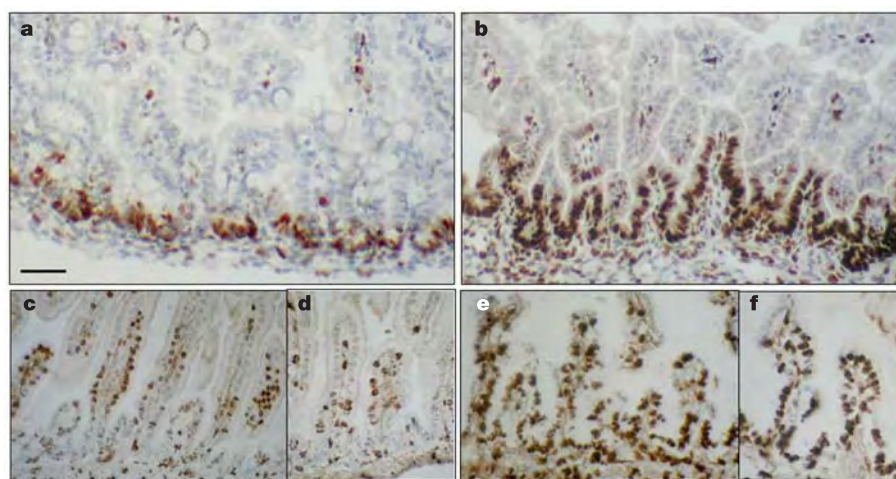


Figure 3 | Activation of Notch expands the population of proliferating intestinal progenitors. Immunohistochemical analysis of sections of small intestine from Rosa-Notch/Cre⁻ (**a**, **c**, **d**) and Rosa-Notch/Cre⁺ (**b**, **e**, **f**) P0 mice stained with an antibody against the proliferating cell antigen Ki67 (**a**, **b**) or with anti-BrdU antibody 24 h after administration of BrdU (**c**–**f**). **a**, **b**, The proliferating cells (brown) are confined to the crypt domain in Rosa-Notch/Cre⁻ intestine (**a**), whereas they are detected all along the villus axis in N1ic-expressing mice (**b**), where only terminally differentiated cells

normally reside. **c**–**f**, BrdU-positive cells (dark brown) have differentiated and migrated upwards in Rosa-Notch/Cre⁻ mice (**c**, **d**), whereas in Rosa-Notch/Cre⁺ sections the number of positively stained cells is increased (**e**, **f**). Scale bars, 80 μ m (**a**, **b**), 60 μ m (**c**–**f**). We note that the morphology of the Rosa-Notch/Cre⁺ sample is inferior to that of the wild-type control, indicating a possibly increased sensitivity of the mutant tissue to the experimental conditions used in these preparations.

Given these findings, we wished to explore the possibility that the differentiation defects observed in Notch transgenic mice were underlined by a mechanism whereby the upregulation of Hes-1 resulted in the repression of the mouse atonal homologue Math-1 (ref. 19) and neurogenin-3 (*ngn3*)²⁰, both coded by essential genes for secretory cell lineage specification. Consistent with this model is our finding that the expression of Math-1 and, to a smaller extent, *ngn3* is repressed by Notch activation (Fig. 2a). We note that the intestinal phenotype we observe is reminiscent of Math-1 knockout mice, which lack goblet and enteroendocrine cells⁸. In contrast, mice lacking Hes-1 activity show an 'opposite' phenotype to that of Math-1 deficient mice, with an excess of secretory cells at the expense of enterocytes⁷. Thus the gain of function phenotype we describe here provides direct evidence that Notch signals target Hes-1 in the intestine, explaining mechanistically the differentiation defects that we observe.

Because it is known that, depending on the cellular context, Notch signalling can significantly influence cell proliferation^{21,22}, we sought to examine the effects of Notch signal activation on cell division in the intestine. Staining intestinal sections from newborn transgenic mice with the proliferation marker Ki67 (ref. 23) reveals a marked increase in proliferating cells in the intervillus regions accompanied by a considerable expansion of the proliferating compartment. In fact, numerous Ki67-positive cells penetrated the villus domain (compare Fig. 3a and b) and we repeatedly detected cells that were still dividing all along the vertical axis of the villi.

This analysis was extended by examining the pattern of migration of bromodeoxyuridine (BrdU)-labelled cells. The intestine of mice expressing intracellular Notch1, killed 24 h after BrdU exposure, displays a marked increase in the number of BrdU-positive cells compared with the *Rosa-Notch/Cre*⁻ littermates (Fig. 3c–f). The distribution of labelled nuclei in the *Rosa-Notch/Cre*⁻ mice reflects the apical migration of cells that have started differentiating after the BrdU pulse (Fig. 3c, d). The noticeable increase in the number of marked nuclei in the *Rosa-Notch/Cre*⁺ mice (Fig. 3e, f) is correlated with the observed pattern of Ki67 expression and furthermore points to an expansion of the population of progenitor cells. However, these experiments do not allow us to determine whether the higher number of BrdU-positive cells is a consequence of an accelerated cell division rate or results from an inhibition of cell cycle arrest at the edge of the prospective crypt region.

To further characterize the nature of the ectopically proliferating cells in the *Rosa-Notch/Cre*⁺ mice, intestinal sections of newborn P0 mice were examined by electron microscopy. Ultrastructural examination of the apical surface of Notch-expressing intestinal epithelial cells revealed a brush border with a lower density of microvilli than in the wild type (Fig. 4). The microvilli of the columnar epithelial cells in *Rosa-Notch/Cre*⁺ villi (Fig. 4b, d) are not uniformly distributed and seem less rigid than those in the *Rosa-Notch/Cre*⁻ littermates (Fig. 4a, c). This phenotype resembles the immature brush border of undifferentiated crypt cells^{24,25}, which is consistent with the notion that Notch activation inhibits differentiation.

Our study provides direct evidence that a major effector of Notch signals in the intestine is Hes-1, in agreement with the previously reported loss of function studies of Hes-1 and its targets^{7,8}. However, Hes-1-deficient mice do not show a change in the proliferative status of the intestinal precursor pool⁷, whereas Notch activation profoundly affects the proliferation potential of intestinal progenitors. In this context, it is worth noting that disruption of Notch signalling by the deletion of the Notch downstream effector CSL (Su(H) in *Drosophila*) results in what can be described as a 'reciprocal' phenotype. All proliferation ceases in the crypt cells, which differentiate towards a secretory fate²⁶. In addition, a recent study in zebrafish uncovered a critical role for Notch in the control of intestinal cell commitment, demonstrating that loss of function mutations in the Notch ligand Delta and block of Notch signals in the *mindbomb*

mutant background result in a dramatic increase of secretory cells at the expense of enterocytes²⁷.

Depletion of crypt epithelial progenitors in mice lacking TCF-4, a downstream effector of the Wnt signalling cascade, have previously shown the importance of the β -catenin/TCF pathway in the maintenance of proliferating crypt cells in the intestine²⁸. Given that the Wnt/Wingless and Notch pathways are known to cross-talk in *Drosophila*²¹, we examined the levels of the Wnt-responsive transcription factors TCF-4 and LEF1 in *Rosa-Notch/Cre*⁺ intestine. We find that Notch activation affects neither the transcription of TCF-4 or LEF1 (Fig. 2a) nor the nuclear localization of β -catenin in the crypt cells (data not shown). These observations are consistent with the epistatic relationship between the Notch and the Wnt pathways postulated in vertebrate somitogenesis, in which LEF1 was found to act upstream of Notch/Delta signalling²⁹.

We consider the observation that we can affect the proliferation potential of all cells in the intestinal crypts to be of particular importance, because it raises the possibility that Notch signals might serve as a tool to affect the number of stem cells known to reside in this region, while inhibiting their differentiation. However, because there are no distinguishing cellular markers for the presumptive gastrointestinal stem cells³⁰, nor do we know the molecular

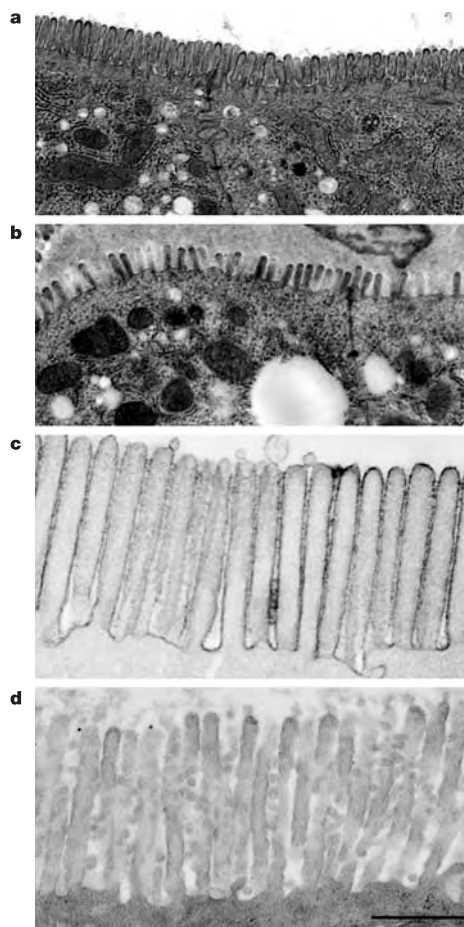


Figure 4 | Apical defect in *Rosa-Notch/Cre*⁺ intestinal epithelial cells. Transmission electron microscopy of the apical surface of intestine from P0 mice. **a, c,** The brush border from *Rosa-Notch/Cre*⁻ epithelial cells reveals densely packed, uniformly distributed microvilli. **b, d,** In *Rosa-Notch/Cre*⁺ intestinal sections, in contrast, there are several areas, along the vertical axis of the villus, with a lower density of brush border microvilli, reminiscent of the immature microvilli seen in undifferentiated crypt cells before their apical migration. Scale bar, 5 μ m (**a, b**), 0.5 μ m (**c, d**).

factors necessary for maintaining the intestinal stem cell niche, it is not possible at this time to determine rigorously whether our transgenic model does indeed affect intestinal stem cells or early precursor cells. Nevertheless, we do know that the villin promoter driving Cre expression¹⁴ targets the intestinal stem cells. Using a mouse that carried a tamoxifen-inducible Cre recombinase, expressed under the control of the villin promoter, we were able to induce transiently the expression of the Cre-responsive Rosa-Notch-IRES-green fluorescent protein (GFP) transgene¹⁵ used in this study. Mice killed 4 months after the tamoxifen induction revealed that the recombined Notch-IRES-GFP allele had persisted in intestinal cells, showing that the stem cell compartment had been targeted¹⁴ (data not shown).

It is clear that a functional characterization of the cells that are expanded after Notch activation will eventually be essential if we are to determine their true nature and developmental fate. We note, however, that developmental plasticity of cells has been intimately associated with proliferation potential and there is therefore the need to examine further the possibility that Notch signals might serve as a tool to influence the mechanisms affecting the number and fate of early precursors, possibly the self-renewal of stem cells in the intestine.

METHODS

Transgenic mice. The Rosa-Notch transgenic mice (provided by C. Murtaugh and D. Melton prior to publication of ref. 15) harbour a construct targeting the intracellular domain of mouse Notch 1 (N1ic) to the ubiquitously expressed Rosa26 locus¹⁵. The N1ic sequence is preceded by a STOP fragment flanked by loxP sites, blocking the expression of N1ic in the absence of Cre recombinase. The N1ic sequence is also followed by internal ribosome entry sequence (IRES) and nuclear enhanced green fluorescent protein (EGFP). When mice carrying this construct are crossed with Cre transgenic mice, recombination occurs at the loxP sites, removing the STOP and allowing heritable coexpression of N1ic and nuclear EGFP. The Villin-Cre mice were generated by S.R. and D.L. and carry a 9-kilobase regulatory region of the mouse villin gene upstream of the Cre recombinase sequence¹⁴. Rosa-Notch homozygous or heterozygous mice were crossed to Villin-Cre/+ mice to obtain Rosa-Notch/Cre⁺ and Rosa-Notch/Cre⁻ progeny. **Western blot analysis.** Tissue extracts were prepared by homogenization in RIPA (radioimmunoprecipitation assay) buffer (1% Triton X-100, 0.5% deoxycholate, 0.1% SDS, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA and a protease/phosphatase inhibitor cocktail). Proteins (100–200 µg) were separated on 6% SDS-PAGE gels, transferred to Immobilon (Millipore) and probed with anti-Notch1 antibody (1/1,000 dilution, a gift from J. Aster). Horseradish peroxidase (HRP)-conjugated secondary antibodies were detected by chemiluminescence.

RT-PCR analysis. Total RNA was isolated from freshly dissected intestine and kidney with Trizol reagent (Life Technologies) and complementary DNA synthesis was performed according to manufacturer's instructions (SuperScript kit; Invitrogen). For PCR amplification reactions, the thermocycler profile used consisted of an initial denaturation at 96 °C for 1 min, followed by 22–28 cycles of 96 °C for 30 s, 55 °C for 30 s and 72 °C for 40 s. The primers used were as follows: mouse *Notch1*, sense 5'-GCTGACCTGCGCATGCTGCCATG-3' and antisense 5'-CATGTTGTCTGGATGTTGGCATCTG-3'; mouse *Hes-1*, sense 5'-ACACCGGACAAACCAAGAC-3' and antisense 5'-GTCACCTCGTTTCATGCATC-3'; *Math1*, sense 5'-GACCACCATCACCTTCGCACCG-3' and antisense 5'-AACTCTCCGTCACCTTCTGTGG-3'; *crypt1*, sense 5'-AAGAGACTAAAAGTCTGAGGAGCAGC-3' and antisense 5'-CGACAGCAGAGCGTGTA-3'; *ngn3*, sense 5'-CGGATGACGCCAACTTACAAAG-3' and antisense 5'-CACAGAAGTCTGAGAACACCAAG-3'; mouse *Hes1*, sense 5'-GAGAAGGCTGGTACCCAGTG-3' and antisense 5'-TGGGATGCGTAGTTGTTGAG-3'; mouse *Hes5*, sense 5'-AGATGCTCAGTCCCAAGGAG-3' and antisense 5'-TAGCCC TCGTGTAGTCTG-3'; *β-actin*, sense 5'-GACGGCCAGGTTCATCTAT-3' and antisense 5'-ACATCTGCTGGAAGGTGGAC-3'; *TCF-4*, sense 5'-CGAGATATCAACGAGGCTTCAAG-3' and antisense 5'-CATGTGATTGCGCTGCGTCTCC-3'; *LEF1*, sense 5'-CTCAACACGACAGAGAAAGGAGCAGG-3' and antisense 5'-GTACCTGAAGTCGACTCCTGTAG-3'.

Histology and immunohistochemistry. Tissues were fixed overnight in 4% neutral-buffered paraformaldehyde at 4 °C, paraffin-embedded and sectioned at 4 µm thickness. Sections were stained with haematoxylin and eosin or subjected to immunohistochemistry with the following primary antibodies: anti-cleaved caspase-3 (1:200 dilution; Cell Signalling), anti-chromogranin A + B (1:100 dilution; Progen), anti-Hes1 (1:500 dilution; a gift from T. Sudo³¹), anti-Ki67

(1:100 dilution; Novocastra), anti-GFP (1:200 dilution; Molecular Probes), anti-BrdU (1:200 dilution; Becton Dickinson) and anti-β-catenin (1:50 dilution; Transduction Laboratories). Antigen retrieval was achieved by boiling in 10 mM citrate buffer pH 6.0 (20 min) for all antibodies, except anti-BrdU, which used treatment with 0.1% trypsin (20 min at 37 °C) and anti-β-catenin, which required boiling in Tris-EDTA (1 mM Tris, 40 mM EDTA) pH 9.0 (20 min). HRP-conjugated secondary antibodies were detected with the diaminobenzidine peroxidase substrate kit (Vector Laboratories). For the detection of nuclear EGFP, fresh tissues were embedded in OTC (optimum cutting temperature) compound (Tissue-Tek), snap-frozen and sectioned on a cryostat at 8 µm thickness. Histochemical identification of intestinal cell types was performed on paraffin sections with periodic acid Schiff, Alcian blue and Grimelius silver stain reagents as recommended by the manufacturer (PolyScientific). BrdU incorporation experiments were performed by injecting pregnant females at day 18.5 of gestation with BrdU (Sigma) at 100 µg per gram animal body weight, dissolved in sterile PBS. After delivery the newborn mice were killed and processed for immunohistochemistry as described.

Electron microscopy. Tissue samples from the proximal tract of the small intestine were fixed in 4% glutaraldehyde in sodium cacodylate buffer (0.1 M, pH 7.4) for 18 h at 4 °C. The tissues were then cut into 5-mm fragments, post-fixed in 1% osmium tetroxide, dehydrated in graded alcohols and embedded in Epon 812. After polymerization overnight at 60 °C, 1-µm sections were prepared and stained with toluidine blue. Representative areas were chosen, thin-sectioned, stained with lead citrate and examined on a Philips 301 electron microscope. Images were captured by an Advanced Microscopy Techniques digital imaging system.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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In vivo imaging of specialized bone marrow endothelial microdomains for tumour engraftment

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The organization of cellular niches is known to have a key role in regulating normal stem cell differentiation and regeneration, but relatively little is known about the architecture of microenvironments that support malignant metastasis^{1,2}. Using dynamic *in vivo* confocal imaging, here we show that murine bone marrow contains unique anatomic regions defined by specialized endothelium. This vasculature expresses the adhesion molecule E-selectin and the chemoattractant stromal-cell-derived factor 1 (SDF-1) in discrete, discontinuous areas that influence the homing of a variety of tumour cell lines. Disruption of the interactions between SDF-1 and its receptor CXCR4 inhibits the homing of Nalm-6 cells (an acute lymphoblastic leukaemia cell line) to these vessels. Further studies revealed that circulating leukaemic cells can engraft around these vessels, suggesting that this molecularly distinct vasculature demarcates a microenvironment for early metastatic tumour spread in bone marrow. Finally, purified haematopoietic stem/progenitor cells and lymphocytes also localize to the same microdomains, indicating that this vasculature might also function in benign states to demarcate specific portals for the entry of cells into the marrow space. Specialized vascular structures therefore appear to delineate a microenvironment with unique physiology that can be exploited by circulating malignant cells.

It is thought that tumour cells derive their ability to transit to specific organs by co-opting the same tissue-homing mechanisms used by benign leukocytes³. Substantial *in vitro* and more limited *in vivo* data provide evidence that tumour cells depend on selectin-, integrin-, and chemokine-mediated vascular cell adhesion events in order to identify and bind to vascular beds at sites of tissue entry^{4,5}. These molecular mechanisms are thought to enable the efficient spread of malignancies to target organs. Differential expression of endothelial signals among tissues is known to control the destination of cellular traffic, but the contributions of the vascular molecular framework to the regulation of complex cellular microenvironments remain to be fully elucidated.

The bone marrow is a common site for the spread of solid tumours. It can also be considered a ubiquitous site for leukaemic cell metastasis, as disease is seen to migrate from the initial birthplace of the leukaemic clone to marrow spaces in distant sites throughout the body. These observations suggest that bone marrow provides a favourable environment for circulating tumour lodgement and growth. Moreover, the bone marrow is commonly the source of latent or 'minimal residual disease' following treatment, raising the possibility that specific anti-apoptotic 'niches' for metastatic growth might exist. Understanding the biological architecture of this host microenvironment therefore has important implications for our approach to tumour treatment.

Although a variety of *in vitro* and *in vivo* techniques exist to study cell transit through the bone marrow, they are limited in their ability to assess the spatial and temporal relationship of cells. To examine the dynamic interactions between intravenously injected tumour cells and the bone marrow microenvironment, we imaged fluorescently labelled cells using *in vivo* confocal microscopy. As the cortex of the mouse skull is relatively thin, imaging of the underlying bone marrow can be performed on the skull with minimal manipulation⁶. Furthermore, the calvarium and other flat bones constitute a large bone marrow compartment, contributing approximately 45% of the hematopoietically active marrow in the adult⁷.

In our initial experiments, we sought to test the hypothesis that leukaemic cells interact with the vascular endothelium in a manner that mimics the multi-step tissue-homing mechanisms of their benign leukocyte counterparts⁸. Progressive scanning and optical sectioning through the bone marrow revealed the existence of unique, spatially restricted vascular domains, at which the majority of marrow-homing Nalm-6 pre-B acute lymphoblastic leukaemia (ALL) cells arrested (Fig. 1a, b). Using video-rate imaging, we observed that leukaemic cells roll along and bind this endothelium minutes after injection (see Supplementary Videos 1–4). Serial imaging of mice on days 3, 10 (Fig. 1c, d) and 14 (data not shown) showed that Nalm-6 cells diapedesed at these sites and increased in numbers in these perivascular locations. This localization of tumour cells to specific regions was not restricted to Nalm-6 cells. Other cell lines, including human (Reh and RS4;11) and murine (300-19) leukaemias, as well as multiple myeloma (U266) and solid tumours (MatLyLu prostatic carcinoma) homed to the same vascular microdomains (see Supplementary Fig. 1).

Given these observations, we hypothesized that the endothelium in these bone marrow regions expresses a unique combination of vascular cell adhesion molecules and/or chemokines capable of attracting a variety of tumour metastases. We first identified the chemokine receptors expressed in common by various cell lines that homed to these regions. Quantitative studies using polymerase chain reaction with reverse transcription (RT-PCR) showed that messenger RNA for the chemokine receptors CXCR3 and CXCR4 is produced by these cells (Fig. 1e). However, flow cytometry experiments detected no cell-surface CXCR3 receptor on Nalm-6 cells, but high levels of cell-surface CXCR4 expression were found (Fig. 1f). *In vitro* transwell chemotaxis assays confirmed that Nalm-6 cells are not responsive to CXCR3 ligands, but do migrate in response to SDF-1, the only chemokine known to bind CXCR4 (Fig. 1g). These studies defined SDF-1 as a candidate chemokine for mediating Nalm-6 homing to these vascular regions. Notably, SDF-1 is

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known to be involved in bone marrow engraftment for a variety of malignant tumour cells, including Nalm-6 cells (refs 9–15).

To examine SDF-1 expression within bone marrow, we performed immunomaging of SDF-1 *in vivo* by labelling cell-surface antigens *in situ* with intravenously injected fluorescent antibodies (J.M.R., X.W., P. Zamiri, D.A.S., A. Bogdanov and C.P.L., unpublished observations). Although SDF-1 is known to be produced by bone marrow stromal cells, mediating B-lymphopoiesis and myelopoiesis in addition to functioning as a chemoattractant, little is known about SDF-1 localization within the marrow space^{16,17}. We found that SDF-1 was expressed in vascular 'hot spots', corresponding to the regions that attract leukaemic cells (Fig. 1h–j, Supplementary Figs 2 and 3). Most of these SDF-1-positive vessels were located parasagittally within the frontal and parietal bones. This parasagittal SDF-1-positive vasculature consisted of sinusoidal networks as well as larger venules leading into these capillary beds. Conversely,

bilateral para-coronal sinusoidal networks, possessing similar size and flow characteristics as the parasagittal sinusoidal beds, were SDF-1-negative. Therefore, SDF-1 expression was not strictly dependent on vessel size or flow.

Using this immunomaging approach, we then examined the regional distributions of various vascular cell adhesion molecules known to be expressed in bone marrow¹⁸. Intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), platelet/endothelial cell adhesion molecule 1 (PECAM-1) and P-selectin were expressed diffusely throughout the marrow vasculature (Fig. 2a–e). However, the pattern of E-selectin expression corresponded to that of SDF-1, and was limited to vessels that supported leukaemic cell engraftment (Fig. 2f–l). E-selectin localization terminated abruptly along individual venules as one moved laterally away from the central axis of the sinusoidal vasculature, again parallel to the pattern seen for SDF-1. Notably, E-selectin expression was

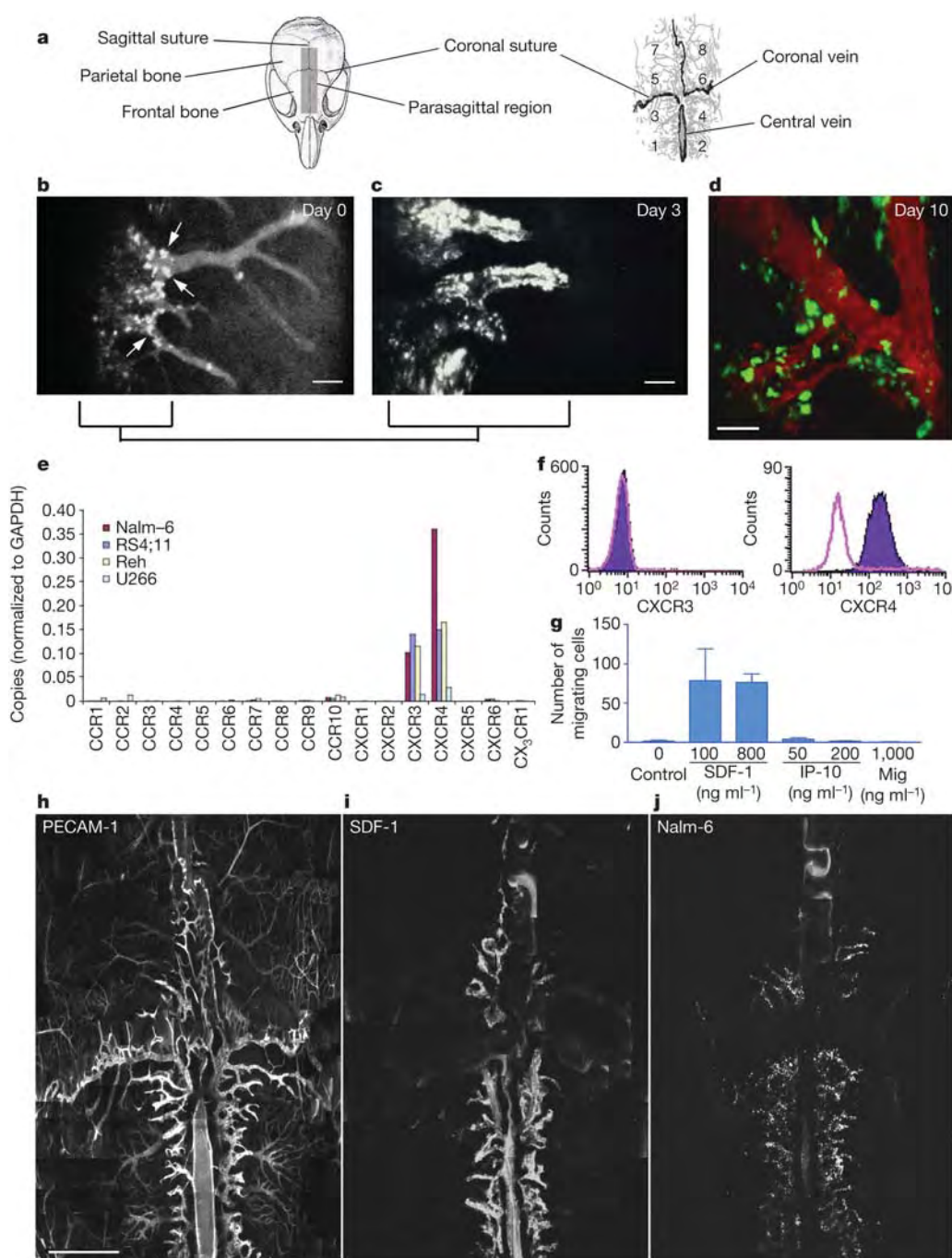


Figure 1 | Leukaemic cell homing and engraftment in mouse skull bone marrow *in vivo*. **a**, Diagrams of the mouse skull and vascular anatomy, divided into quadrants for reference. **b**, Nalm-6 cells bound to parasagittal vascular segments (arrows) 1 h after injection (area 4). Scale bar, 200 μ m. **c**, **d**, Peri-vascular cell growth 3 and 10 days after injection. Scale bars, 100 μ m. **e**, Quantitative RT-PCR of Nalm-6 and other tumour cell lines shows abundant mRNA levels for CXCR4 and CXCR3. Gene expression copy numbers have been normalized to the copy number of the housekeeping gene GAPDH for each cell line. **f**, Flow cytometry of Nalm-6 cells shows cell-surface expression of CXCR4 only. **g**, *In vitro* chemotaxis of Nalm-6 cells in response to the CXCR4 ligand SDF-1, but not the CXCR3 ligands IP-10 or Mig. Error bars represent standard error of the mean. **h**, Bone marrow vasculature labelled with anti-CD31/PECAM (assembled from approximately 500 images; scale bar, 1 mm). Signal dampening in lateral regions is due to variations in tissue optical properties (see Supplementary Fig. 10). **i**, Montage image assembled from a different mouse showing restricted SDF-1 expression (see also Supplementary Fig. 3, PECAM-1/SDF-1 co-label). **j**, Nalm-6 cell homing to SDF-1-expressing regions in a third mouse.

dramatically demarcated, to the extent that E-selectin was expressed on only one wall of certain microvessels (Fig. 2g–i). We confirmed these results by repeating our *in vivo* immunofluorescence studies of PECAM-1, SDF-1 and E-selectin expression using alternative antibody clones (see Methods for details).

Together, the immunofluorescence experiments suggest that specific microvascular domains in the bone marrow express high levels of both E-selectin and SDF-1. *In vivo* co-localization studies confirmed that Nalm-6 cell binding was restricted to these same E-selectin⁺ SDF-1⁺ vessel beds (Fig. 3a–f). These observations raise the possibility that E-selectin and/or SDF-1 regulate this restricted leukaemic cell homing.

To test this hypothesis, we evaluated the functional role of E-selectin and CXCR4–SDF-1 interactions for leukaemic homing to these specific regions. When Nalm-6 cells were injected into an E-selectin knockout mouse, we observed a <20% reduction in their homing to bone marrow (Fig. 3g, h, k). In contrast, when cells were pre-treated with pertussis toxin, an inhibitor of chemokine receptor Gα_i-mediated signalling, homing was decreased by almost 80% (Fig. 3k). Similar results were obtained upon CXCR4 desensitization and downregulation in response to SDF-1 (Fig. 3k and Supplementary Fig. 4), as well as highly specific CXCR4 receptor blockade by the small molecule inhibitor AMD3100 (ref. 19; Fig. 3i–k). These data suggest that although E-selectin might serve to enhance homing, the majority of Nalm-6 cell vascular adhesion events can occur in its absence. Loss of SDF-1, however, does not appear to be compensated for by other adhesive molecules. These results also suggest that SDF-1–CXCR4 interactions, known to be important for cell retention in the marrow matrix (see also Supplementary Fig. 5), are pivotal at the earliest stage of tumour metastasis in the bone marrow (recognition and binding to permissive vasculature)²⁰. Although this

mechanism has been suggested by *in vitro* data, it has not previously been observed *in vivo*^{21,22}.

We next examined whether cells that were prevented from homing to the bone marrow spread to alternative tissue compartments or remained in the peripheral circulation. To quantify leukaemic cell homing kinetics, we used *in vivo* flow cytometry, a new technology for detecting and counting individual, fluorescently labelled cells flowing through peripheral vessels as a function of time post-injection²³. As shown in Fig. 3l, >70% of Nalm-6 cells exit the circulation in control mice in the first hour after injection, consistent with the rapid homing to bone marrow observed using microscopy. In contrast, <20% of cells treated with AMD3100 exit the circulation within the first hour, suggesting that cells inhibited from bone marrow homing remain in the peripheral circulation. The cells exit the circulation with a time course consistent with when the drug effect is expected to decline²⁴.

Last, we explored the normal function of these vascular regions. Because SDF-1 is known to be a potent chemoattractant for haematopoietic stem/progenitor cells (HSPCs) and lymphocytes, and as blood-borne HSPCs and lymphocytes traffic to bone marrow, we hypothesized that circulating HSPCs and lymphocytes might preferentially home to these same microdomains^{25,26}. Fluorescently labelled HSPCs isolated from donor BALB/c mice were injected into recipient mice, and intravital bone marrow imaging was performed at multiple time points. Representative images taken 2 h after injection show HSPCs adherent to bone marrow microvasculature in the same restricted domains as SDF-1 vascular expression (Fig. 4a–c). Seventy days after injection (Fig. 4d), populations of labelled cells are still detected in the bone marrow extravascular space within these microdomains. Although we cannot exclude the possibility that the observed fluorescence signal comes from cell debris that has been

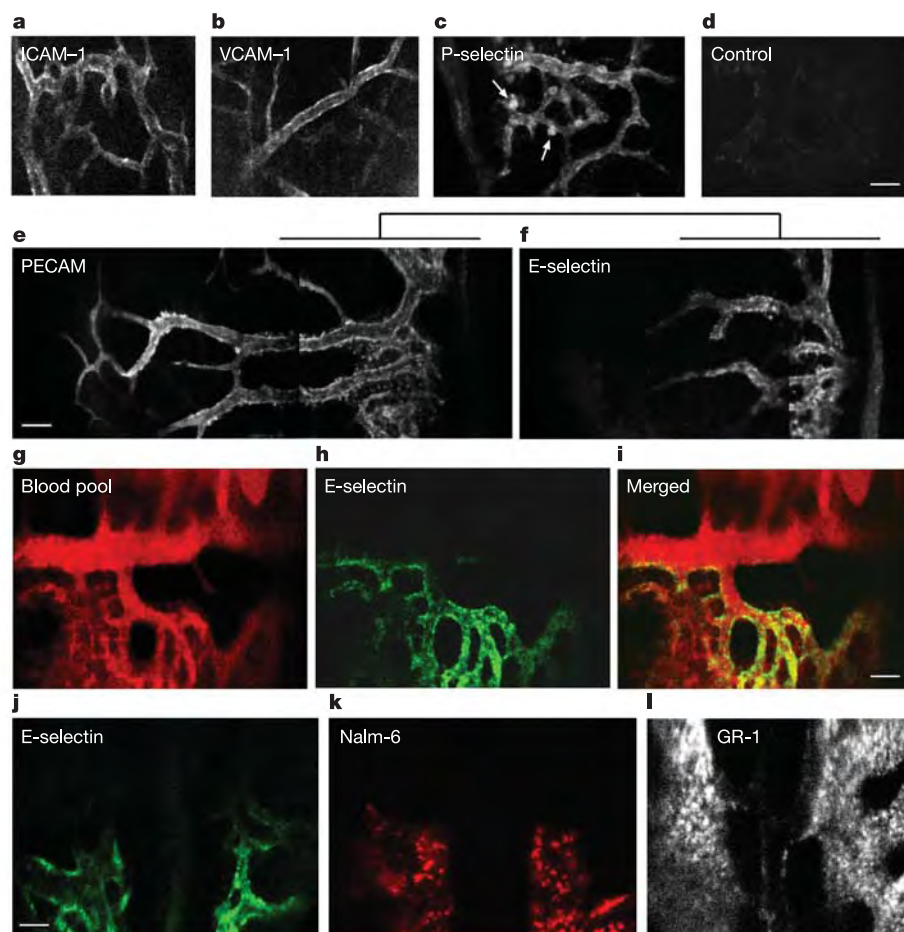


Figure 2 | *In vivo* immunofluorescence microscopy of vascular cell adhesion molecule expression in bone marrow. a–d, Representative images of ICAM-1 (a, area 6), VCAM-1 (b, area 8), P-selectin (c, area 4) and isotype control staining (d). ICAM-1, VCAM-1 and P-selectin are expressed throughout the bone marrow vasculature. Arrows in c indicate P-selectin-positive megakaryocytes. e, f, Montage images of extended PECAM-1 expression compared with restricted E-selectin expression (both area 3). g–i, An image series from area 4/6. The bone marrow blood pool (red) is delineated by a non-specific fluorescent antibody signal. E-selectin expression (green) is restricted to one wall of a large collecting vein and to sinusoidal microvasculature in area 4. j–l, E-selectin expression (green) corresponds with Nalm-6 cell (red) homing patterns. The vascular staining pattern is contrasted with anti-GR1 (myeloid differentiation antigen) staining of the same region (midline, intersection of areas 3–6). Scale bars in i and j, 100 μ m.

phagocytosed by other cells (for example, macrophages), the morphology and persistence of dye are consistent with the engraftment of a primitive cell or stem cell pool with slow cell cycling¹. Flow cytometry studies of cultured cells confirmed the stability of membrane dye signal in slowly dividing cells, while rapidly dividing cells diluted dye to undetectable levels after 4–5 cell divisions (see Supplementary Fig. 6). In another set of experiments, T lymphocytes isolated from the spleen and lymph nodes of a BALB/c mouse were labelled and injected intravenously into a SCID recipient (see Supplementary Fig. 7). Again, cells immediately homed to the

same microdomains, and were observed within these areas two weeks later. These observations suggest that benign circulating HSPCs, mature T lymphocytes and leukaemic cells use the same vascular subregions for localization in bone marrow. Whether these unique vascular domains intersect or interact with the previously defined bone marrow endosteal cellular niche for haematopoietic stem cells has not yet been defined. Whether benign and malignant cells use overlapping or distinct molecular features to identify these vascular sites is the subject of ongoing experiments; however, our preliminary data show that homing of benign T lymphocytes to the bone marrow is only minimally inhibited by AMD3100 or CXCR4 blockade. Moreover, results from other investigators suggest that treatment with AMD3100 only modestly inhibits HSPCs from homing to bone marrow²⁷. Should differences in how these and other benign and malignant cells recognize bone marrow microenvironments be confirmed, they could be exploited to thwart metastasis while minimizing effects on normal bone marrow function.

It has long been believed that early micrometastases from liquid and solid tumours occur in specific anatomic compartments of the bone marrow, although no molecular basis for such regionalization has been established²⁸. Although many investigations have explored the roles of various cell adhesion molecules and chemokines in cell homing to the bone marrow, studies based on traditional methods have been limited by spatial and temporal sampling restrictions. Elegant studies using bioluminescence imaging have provided temporal information, but are restricted by limitations of spatial resolution²⁹. Our approach has allowed us to combine a high degree of spatial and temporal resolution with an *in vivo* molecular labelling technique. We have shown that the bone marrow microvasculature is comprised of functional domains that are characterized by the expression of specific vascular cell-surface adhesion molecules and chemoattractants crucial for malignant cell homing to these areas. In a benign setting, these domains also appear to define sites at which circulating haematopoietic stem/progenitor cells and lymphocytes enter the bone marrow. Of note, other molecules that are more diffusely expressed might still contribute to the localized homing and engraftment processes that we have observed. Our *in vivo* imaging and flow cytometry approaches should be widely

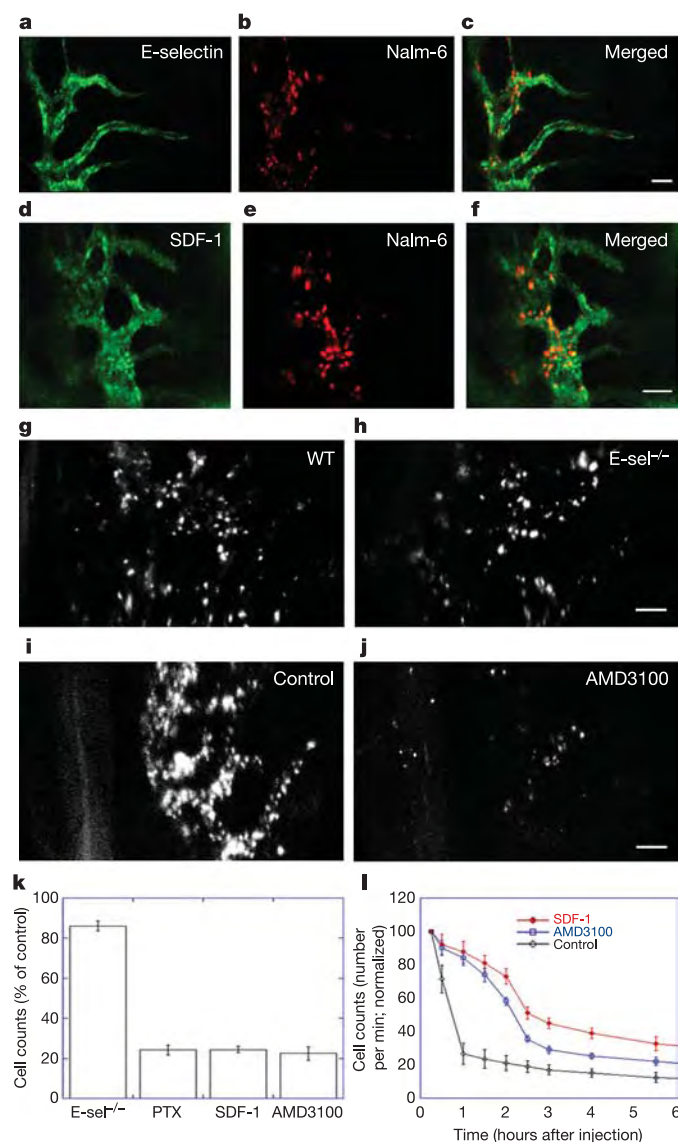


Figure 3 | Leukaemic cell homing to SDF-1⁺E-selectin⁺ microvascular domains is inhibited by SDF-1/CXCR4 blockade. **a–c**, Co-localization of E-selectin expression (green) and Nalm-6 cell homing (red, 30 min after injection) in the parasagittal region of area 4. **d–f**, Co-localization of SDF-1 vascular expression (green) and Nalm-6 cell homing (red, 30 min after injection) in area 3. **g, h**, Cell homing is slightly decreased (<20%) in the E-selectin knockout mouse (E-selectin^{-/-}) compared with wild-type (WT) control (1 h after injection). **i, j**, Nalm-6 cell homing is inhibited by AMD3100 blockade of CXCR4. **k**, The effect of pertussis toxin (PTX) treatment, SDF-1-induced CXCR4 desensitization³⁰, and AMD3100 treatment on cell homing to bone marrow microdomains. **l**, *In vivo* flow cytometry experiments show that Nalm-6 cells blocked from bone marrow homing by SDF-1-induced CXCR4 desensitization or a single dose of AMD3100 remain in the peripheral circulation. As the treatment effects wear off, cells exit the circulation. Error bars in **k** and **l** represent s.e.m. Scale bars in **a–j**, 100 μ m.

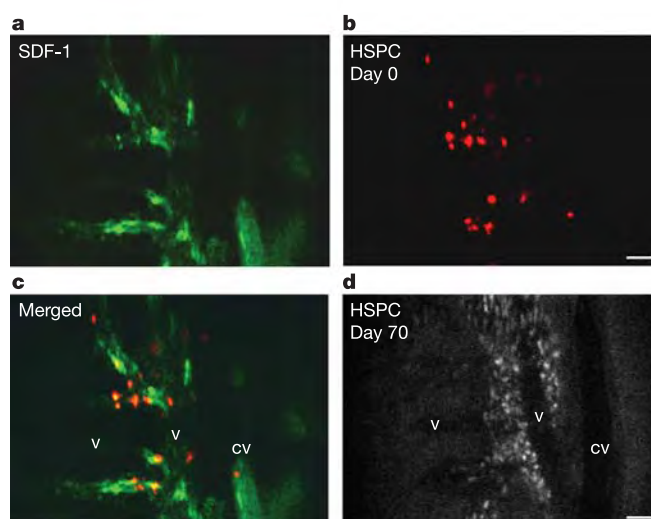


Figure 4 | HSPCs home to SDF-1-positive vascular microdomains. **a–c**, Co-localization of SDF-1 (green) and HSPCs (red) 2 h after injection (parasagittal region of area 3). **d**, An image from the same mouse, taken 70 days after HSPC injection (approximately the same area as in **a–c**). Although fluorescence signal intensity is decreased owing to HSPC proliferation, engrafted cells are apparent in the perivascular spaces. Scale bars, 100 μ m. CV, central vein; v, venule.

applicable to the study of these and other molecules (see Supplementary Figs 8 and 9).

We do not yet know whether the observed patterns of molecular expression represent a response to ongoing signals from adjacent stromal or haematopoietic elements, or whether they reflect embryologic patterning. Although we have focused our studies on the calvarial flat bone, the molecular and functional regionalization of bone marrow vasculature and the ability of these domains to sustain cell engraftment might not be identical in all marrow spaces. As flat and long bones form through different processes (intramembranous versus endochondral ossification), it will be of interest to investigate whether marrow cavities that arise through different developmental pathways show unique molecular patterning of the vasculature.

As these bone marrow vascular gateways also appear to denote favourable environments for tumour engraftment, their targeted blockade could protect the marrow from continued metastasis in settings where residual disease is suspected. Greater understanding of how these regions might foster cell growth, and whether malignant and benign cells compete for entry or survival within bone marrow microdomains, could thus provide new targets for intervention in the treatment of metastatic disease.

METHODS

BALB/c or SCID mice were anesthetized and a small incision made in the scalp so as to expose the underlying dorsal skull surface. For cell tracking, 5×10^6 leukaemic cells labelled with the fluorescent lipophilic tracers DiD or DiR (Molecular Probes) were injected into the tail vein. Leukaemic cell homing to bone marrow vasculature of the skull was then imaged using a custom-built fluorescence confocal/multiphoton microscope while the mice were under anaesthesia on a warmed microscope stage. For *in vivo* immunofluorescence imaging, antibodies were either purchased as fluorescent conjugates or were conjugated to fluorescent cyanine compounds (Amersham) or to AlexaFluor750 (Molecular Probes) according to the manufacturers' protocols. Labelled anti-vascular cell adhesion or isotype control antibodies were injected into mice through the tail vein at doses of $0.5\text{--}1\text{ mg kg}^{-1}$. Approximately 24 h were allowed to elapse before imaging in order to maximize antibody binding and clearance of unbound label from the circulation. High-resolution images with cellular details were obtained through the intact mouse skull at depths of up to $250\text{ }\mu\text{m}$ from the surface of the skull using an $\times 30$ 0.9NA water immersion objective lens. Quantitative evaluation was made by dividing the bone marrow into pre-determined quadrants and counting the number of fluorescent cells per field.

Please refer to the Supplementary Methods for descriptions of the imaging system and the *in vivo* flow cytometer, and for additional information on cell purification, cell labelling, vascular CAM immunostaining, serial imaging, colocalization, quantitative PCR, chemotaxis assays, CXCR4 desensitization by SDF-1 and CXCR4 blockade by AMD3100.

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LETTERS

Stem cell division is regulated by the microRNA pathway

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One of the key characteristics of stem cells is their capacity to divide for long periods of time in an environment where most of the cells are quiescent. Therefore, a critical question in stem cell biology is how stem cells escape cell division stop signals. Here, we report the necessity of the microRNA (miRNA) pathway^{1–4} for proper control of germline stem cell (GSC) division in *Drosophila melanogaster*. Analysis of GSCs mutant for *dicer-1* (*dcr-1*), the double-stranded RNaseIII essential for miRNA biogenesis, revealed a marked reduction in the rate of germline cyst production. These *dcr-1* mutant GSCs exhibit normal identity but are defective in cell cycle control. On the basis of cell cycle markers and genetic interactions, we conclude that *dcr-1* mutant GSCs are delayed in the G1 to S transition, which is dependent on the cyclin-dependent kinase inhibitor Dacapo, suggesting that miRNAs are required for stem cells to bypass the normal G1/S checkpoint. Hence, the miRNA pathway might be part of a mechanism that makes stem cells insensitive to environmental signals that normally stop the cell cycle at the G1/S transition.

MicroRNAs and short interfering RNAs (siRNAs), processed by the type III double-stranded RNase Dicer, function in an RNA-based mechanism of gene silencing^{1–4}. Most characterized miRNAs from animals repress gene expression by blocking the translation of complementary messenger RNAs into protein; they interact with

their targets by imperfect base-pairing to mRNA sequences within the 3' untranslated region (3' UTR)¹. Experimental evidence has suggested that small RNAs regulate stem cell character in plants and animals^{5–7}. Moreover, some miRNAs are differentially expressed in stem cells, suggesting a specialized role in stem cell regulation^{8,9}. However, the molecular mechanisms underlying stem cell control by miRNAs are not understood.

To determine the role of miRNAs in the control of stem cell biology, we specifically eliminated processing of all miRNAs in stem cells. The *Drosophila* genome contains two Dicer isozymes: Dicer-1 and Dicer-2 (ref. 10). Dicer-1 (Dcr-1) is essential for processing miRNAs, whereas Dicer-2 (Dcr-2) is required for siRNAs; loss of Dcr-1 completely disrupts the miRNA pathway and only has a weak effect on the siRNA pathway. Using *Drosophila* GSCs as a model system, we impaired Dcr-1 activity with two *dcr-1* alleles: *dcr-1*^{d102} and a null *dcr-1*^{Q1147X} (ref. 10). *Drosophila* oogenesis depends on the presence of self-renewing GSCs in the adult ovary^{11,12}, as has recently been reported in a mammalian system¹³. The continuous division of GSCs generates an array of progressively developed egg chambers in wild-type ovarioles (Fig. 1).

Analysis of *dcr-1* mutant clones in the *Drosophila* ovary 12 days after clone induction revealed a marked depletion of developing egg chambers (see Fig. 1b–f for *dcr-1*^{Q1147X} and Supplementary Fig. 1a

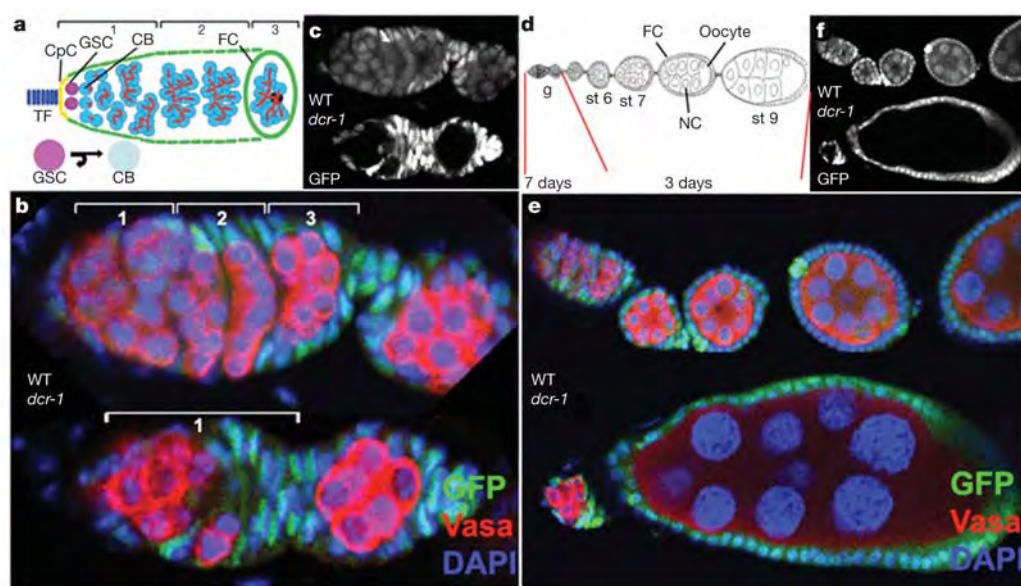


Figure 1 | Loss of Dcr-1 function in GSCs reduces the rate of egg chamber production.

a, Schematic of a germarium divided into three regions. Region one contains GSCs and dividing cysts. **b, c**, All three regions are observed in a wild-type heterozygous *dcr-1*^{Q1147X/+} germarium (WT, top), but not in a mosaic *dcr-1*^{Q1147X} germarium 12 days after clone induction (no GFP, *dcr-1*, bottom). **d**, Oocyte development is divided into 14 stages. **e, f**, Many of the developmental stages are missing in ovarioles that are complete *dcr-1*^{Q1147X} germline clones (no GFP, 12 days after clone induction; bottom of panels **e** and **f**). CpC, cap cells; CB, cystoblast; DAPI, 4,6-diamidino-2-phenylindole; FC, follicle cells; NC, nurse cells; TF, terminal filaments. Vasa marks the germ line. Absence of GFP marks *dcr-1* mutant cells.

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for *dcr-1*^{d102}). In contrast, *dcr-2* null mutant GSCs produced a normal progression of egg chambers. These data suggest that Dcr-1 is required for efficient germline production. Although *dcr-1* mutants showed reduced numbers of gametes, most developing gametes appeared morphologically normal (although they exhibit polarity defects; data not shown). We therefore analysed potential problems in GSC maintenance, identity and division. Clonal experiments revealed that the percentage of germaria with clonal stem cells at different time points after clone induction was similar in the *dcr-1*^{Q1147X} mutant and the wild-type control (Fig. 2b), suggesting that the loss of cysts in *dcr-1* mutants is not primarily due to problems in the maintenance of GSCs.

To determine whether reduced cyst production in *dcr-1* germaria was due to altered GSC fate, we analysed the identity of the *dcr-1* mutant GSCs. Female GSCs are identified by their location and the expression patterns of three markers (Fig. 2a): the presence of Adducin, a protein present in the spectrosome¹⁴; the presence of phosphorylated Mad protein (P-Mad), indicating TGF- β signalling^{14,15}; and the absence of Bam, repressed by the TGF- β pathway¹⁶. The *dcr-1*^{Q1147X} GSCs showed normal spectrosome morphology and position (100%, $n = 53$), and normal TGF- β pathway activity (P-Mad:

wild type 88%, $n = 114$; *dcr-1*^{Q1147X} 85%, $n = 47$; Fig. 2c, d). Furthermore, as with wild-type GSCs, *dcr-1*^{Q1147X} GSCs did not stain positively for the Bam protein (Fig. 2e). From these analyses, we conclude that decreased cyst production from *dcr-1*^{Q1147X} GSCs does not result from either a loss of GSCs or a change in their identity.

The frequency of cell division in *dcr-1*^{Q1147X} GSCs was impaired. Examination of individual germaria containing a single heterozygous GSC and a single *dcr-1*^{Q1147X} mutant GSC revealed that GSCs lacking Dcr-1 activity produced cysts at a frequency that was reduced to 18% of normal levels (41% for *dcr-1*^{d102}; Table 1 and Fig. 3a–c). In contrast, the frequency of division was not significantly reduced for GSCs that were homozygous for the *dcr-2* mutation or for the isogenized parental chromosome from which the *dcr-1* mutant alleles were generated (Table 1 and Fig. 3c). Thus, Dcr-1 is required cell autonomously in GSCs for the cell divisions that produce developing cystoblasts (no obvious defect in cyst division was observed; see Supplementary Fig. 2c, d).

To determine whether the reduced cyst formation reflected a block in the normal cell cycle programme, we analysed the distribution of cell cycle stages in mutant *dcr-1*^{Q1147X} GSCs by staining mosaic germaria with antibodies against different cell cycle markers (Fig. 3d).

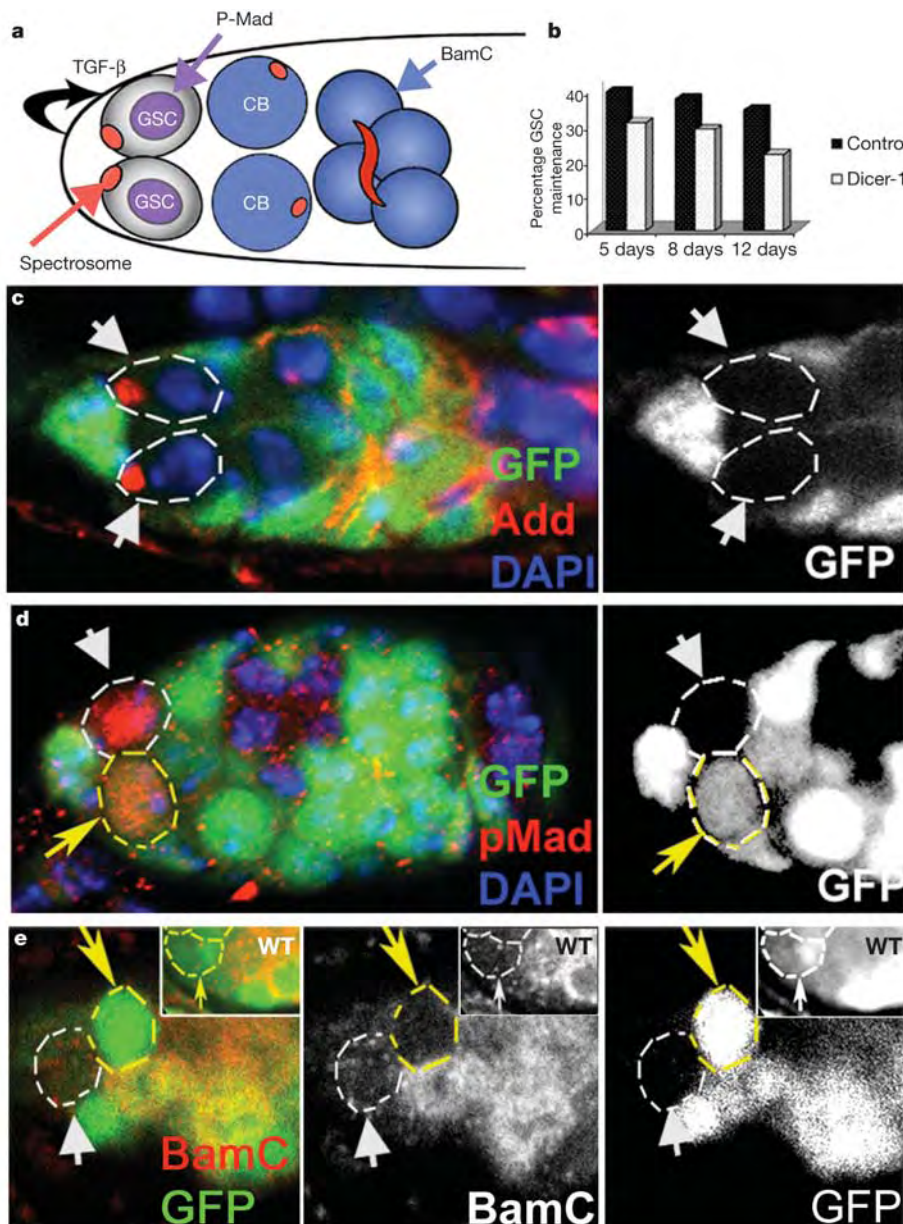


Figure 2 | *dcr-1* mutant GSCs remain in the stem cell niche and retain stem cell identity. **a**, GSC markers: P-Mad, spectrosome (Adducin, Add), lack of BamC. **b**, *dcr-1*^{Q1147X} mutant and wild-type GSCs are maintained in germaria. A low rate of GSC loss was observed for both backgrounds³⁰. Bars represent the percentage of germaria with clonal stem cells at different time points after clone induction. **c–e**, *dcr-1*^{Q1147X} GSCs (no GFP, white arrows) possess a spectrosome (100%, $n = 53$; **c**) and respond to the TGF- β signal (anti-P-Mad staining: *dcr-1* mutant, 85%, $n = 47$; control, 88%, $n = 114$; **d**) but do not exhibit BamC expression ($n = 13$; **e**). Yellow arrows in **d**, **e** mark non-clonal, wild-type GSCs.

Table 1 | Effect of the miRNA pathway on GSC division frequency

Brief genotype	Genotype of GSC and cysts	Frequency of GSC division ($\pm$ s.d.)*	Sample size (n)	Division index ($\pm$ s.d.)†
Wild type	Non-clonal (GFP ⁺): <i>hsFlp; FRT82B hsNmyc/FRT82B Ubi-GFP</i>	4.53 $\pm$ 0.93	17	-
Wild type	Clonal (GFP ⁻): <i>hsFlp; FRT82B hsNmyc/FRT82B hsNmyc</i>	4.64 $\pm$ 0.87	17	1.03 $\pm$ 0.20
<i>dcr-1^{Q1147X}</i>	Non-clonal (GFP ⁺): <i>hsFlp; FRT82B dcr-1^{Q1147X}/FRT82B Ubi-GFP</i>	4.37 $\pm$ 0.74	27	-
<i>dcr-1^{Q1147X}</i>	Clonal (GFP ⁻): <i>hsFlp; FRT82B dcr-1^{Q1147X}/FRT82B dcr-1^{Q1147X}</i> (8 days)	1.41 $\pm$ 0.69	27	0.32 $\pm$ 0.16
<i>dcr-1^{Q1147X}</i>	Clonal (GFP ⁻): <i>hsFlp; FRT82B dcr-1^{Q1147X}/FRT82B dcr-1^{Q1147X}</i> (12 days)	0.77 $\pm$ 0.59	20	0.18 $\pm$ 0.13
<i>dcr-1^{d102}</i>	Non-clonal (GFP ⁺): <i>hsFlp; FRT82B dcr-1^{d102}/FRT82B Ubi-GFP</i>	4.64 $\pm$ 0.83	24	-
<i>dcr-1^{d102}</i>	Clonal (GFP ⁻): <i>hsFlp; FRT82B dcr-1^{d102}/FRT82B dcr-1^{d102}</i> (8 days)	2.5 $\pm$ 0.65	24	0.53 $\pm$ 0.26
<i>dcr-1^{d102}</i>	Clonal (GFP ⁻): <i>hsFlp; FRT82B dcr-1^{d102}/FRT82B dcr-1^{d102}</i> (12 days)	1.72 $\pm$ 0.56	39	0.41 $\pm$ 0.13
<i>dcr-2</i>	Non-clonal (GFP ⁺): <i>hsFlp; FRT42D dcr-2/FRT42D Ubi-GFP</i>	4.48 $\pm$ 1.31	24	-
<i>dcr-2</i>	Clonal (GFP ⁻): <i>hsFlp; FRT42D dcr-2/FRT42D dcr-2</i> (8 days)	4.01 $\pm$ 0.88	24	0.89 $\pm$ 0.19
<i>dcr-2</i>	Clonal (GFP ⁻): <i>hsFlp; FRT42D dcr-2/FRT42D dcr-2</i> (12 days)	4.20 $\pm$ 1.36	24	0.85 $\pm$ 0.28
Parental chromosome	Non-clonal (GFP ⁺): <i>hsFlp; FRT82B (parental)/FRT82B Ubi-GFP</i>	4.58 $\pm$ 0.97	23	-
Parental chromosome	Clonal (GFP ⁻): <i>hsFlp; FRT82B (parental)/FRT82B (parental)</i> (8 days)	4.80 $\pm$ 1.04	23	1.04 $\pm$ 0.17
Parental chromosome	Clonal (GFP ⁻): <i>hsFlp; FRT82B (parental)/FRT82B (parental)</i> (12 days)	5.77 $\pm$ 0.69	22	1.16 $\pm$ 0.14
<i>dap</i>	Non-clonal (GFP ⁺): <i>hsFlp; FRT42B dap⁴/FRT42B Ubi-GFP</i>	4.44 $\pm$ 0.66	20	-
<i>dap</i>	Clonal (GFP ⁻): <i>hsFlp; FRT42B dap⁴/FRT42B dap⁴</i>	4.12 $\pm$ 0.79	20	0.93 $\pm$ 0.18
<i>dap/+; dcr-1</i>	Non-clonal (GFP ⁺): <i>hsFlp; FRT42B dap⁴/+; FRT82B dcr-1^{Q1147X}/FRT82B Ubi-GFP</i>	4.25 $\pm$ 0.55	29	-
<i>dap/+; dcr-1</i>	Clonal (GFP ⁻): <i>hsFlp; FRT42B dap⁴/+; FRT82B dcr-1^{Q1147X}/FRT82B dcr-1^{Q1147X}</i>	2.72 $\pm$ 0.75	19	0.64 $\pm$ 0.18

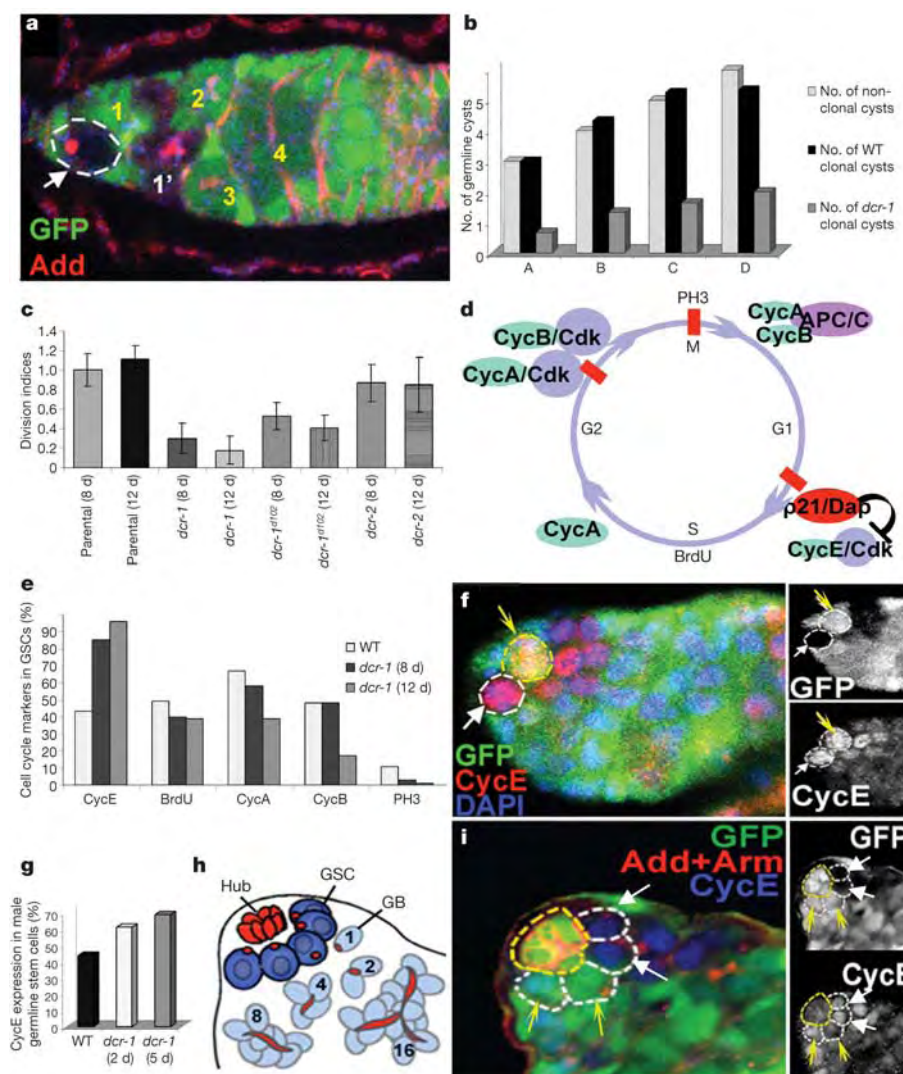
8 or 12 days indicate the number of days after clone induction (this is 8 days if not indicated).

*The number of cystoblasts and cysts divided by the number of GSCs.

†The frequency of clonal GFP⁻ GSC division divided by the frequency of control non-clonal GFP⁺ GSC division.

We observed an increase in the number of *dcr-1* mutant GSCs staining positive for Cyclin E (CycE) using two independent *dcr-1* alleles (Fig. 3e, f; see also Supplementary Table 1 and Supplementary Fig. 1b). In contrast, GSCs that were homozygous for *dcr-2* or the

parental chromosome expressed CycE with frequencies similar to that of wild-type GSCs (Supplementary Fig. 1b). Furthermore, pulse labelling of ovaries with the nucleotide analogue 5-bromo-deoxyuridine (BrdU)^{17,18} revealed that the number of *dcr-1^{Q1147X}*

**Figure 3 | *dcr-1* causes a cell cycle delay in**

GSCs. **a**, *dcr-1^{Q1147X}* GSCs (white arrow) produce fewer cysts (white number) than wild-type GSCs (yellow numbers). **b**, *dcr-1^{Q1147X}* cysts are produced at a lower frequency than wild-type and non-clonal cysts. Samples A–D are grouped according to the number of non-clonal cysts per germlarium ($n = 27$). **c**, Division indices are decreased for *dcr-1* mutant GSCs compared with control and *dcr-2* mutant GSC clones. Error bars represent standard deviation. Times are days after the last clonal induction. **d**, Cell cycle markers. **e**, *dcr-1^{Q1147X}* (*dcr-1*) GSCs are more frequently CycE-positive and less frequently positive for other cell cycle markers compared with wild-type *dcr-1^{Q1147X}/+* GSCs. **f**, CycE-positive *dcr-1* mutant GSC (white arrow). **g**, Percentage of CycE-positive male GSCs that are *dcr-1^{Q1147X}* or wild type. **h**, Male GSC niche. **i**, CycE in male *dcr-1* mutant GSCs (white arrow). Arm (Armadillo) marks somatic hub cells. Yellow arrows in **f**, **i** mark non-clonal, wild-type GSCs.

mutant GSCs in S phase was reduced (Fig. 3e; see also Supplementary Table 1). Similarly, the number of *dcr-1*^{Q1147X} mutant GSCs staining positive for Cyclin A (CycA), Cyclin B (CycB) and the mitotic marker Phosphohistone-3 (PH3) was reduced (Fig. 3e; see also Supplementary Table 1). These data indicate that perturbation of the miRNA pathway by mutant *dcr-1* in GSCs delays the cell cycle at the G1/S transition.

We tested whether loss of Dcr-1 function has similar consequences on the cell cycle in the GSCs of male flies. Each male testis contains approximately ten GSCs surrounding a somatic structure called the hub (Fig. 3h–i)^{19,20}. Similar to female GSCs, the number of male GSCs staining positive for CycE was increased in *dcr-1* mutants (Fig. 3g–i). These data show that Dcr-1 also functions in the male GSC niche, and suggest that Dcr-1 has a conserved role in GSC division.

To test the possibility that the miRNA pathway might be a general cell cycle regulator, we examined other cell types to determine whether the G1/S delay and reduced cell division frequency are also observed in other mitotically dividing *dcr-1* mutant cells. *dcr-1*^{Q1147X} clones in imaginal discs revealed that the number of CycE-positive cells was not increased in mutant cells (Supplementary Fig. 2a). The number of *dcr-1*^{Q1147X} mutant cells in imaginal discs was approximately equal to the number of marked wild-type cells that descended from a common parent cell, indicating that the frequency of cell division in imaginal disc cells is not reduced in a *dcr-1* mutant (Supplementary Fig. 2a, b). *dcr-1*^{Q1147X} dividing germline cysts express CycE at a frequency comparable to that of wild-type dividing cysts, suggesting that the mitotic cystoblast cell divisions are not affected in *dcr-1* mutants (Supplementary Fig. 2c, d). Therefore,

the reduction in cell division frequency observed in the *dcr-1* mutant germ line is specific to the GSC division. Together, these data suggest that the miRNA pathway has a specific role in regulating stem cell division.

We explored the potential cause for the G1/S arrest by examining the expression of Dacapo (Dap; a homologue of the p21/p27 family of cyclin-dependent kinase (CDK) inhibitors)^{21,25} in *dcr-1*^{Q1147X} mutant GSCs. The transition between the G1 and S phases of the cell cycle is negatively regulated by Dap^{21,25}. Dap protein traps the CycE/CDK2 complex in a stable but inactive form²², and elevated levels of Dap lead to cell cycle arrest at the G1/S phase transition with prolonged expression of CycE protein¹⁷. Notably, the number of Dap-positive GSCs increased in the *dcr-1* mutant GSC population (Fig. 4b; see also Supplementary Table 1 and Supplementary Fig. 3a).

To determine whether Dap mediated the effect of *dcr-1* on the GSC cell cycle, we reduced the level of Dap by 50% in *dcr-1*^{Q1147X} mutant GSCs and observed a partial rescue in cyst production (Table 1 and Fig. 4c, e). Furthermore, the number of GSCs staining positive for CycE was reduced to normal levels (Fig. 4d), demonstrating that the CycE defect observed in *dcr-1* mutant GSCs is dependent on Dap. Consistent with this, overexpression of a Dap transgene resulted in some germlaria resembling *dcr-1* germline mutants: the germlaria were small, containing a few cysts, and had a high number of CycE-positive GSCs (Fig. 4d; see also Supplementary Fig. 3d). The fact that reduction of Dap levels led to a normal GSC CycE profile, but partial rescue of cyst generation, suggests that Dcr-1 might also regulate later cyst development.

These data suggest that miRNAs act on stem cell division by reducing the levels of Dap. How is this regulation achieved? We found

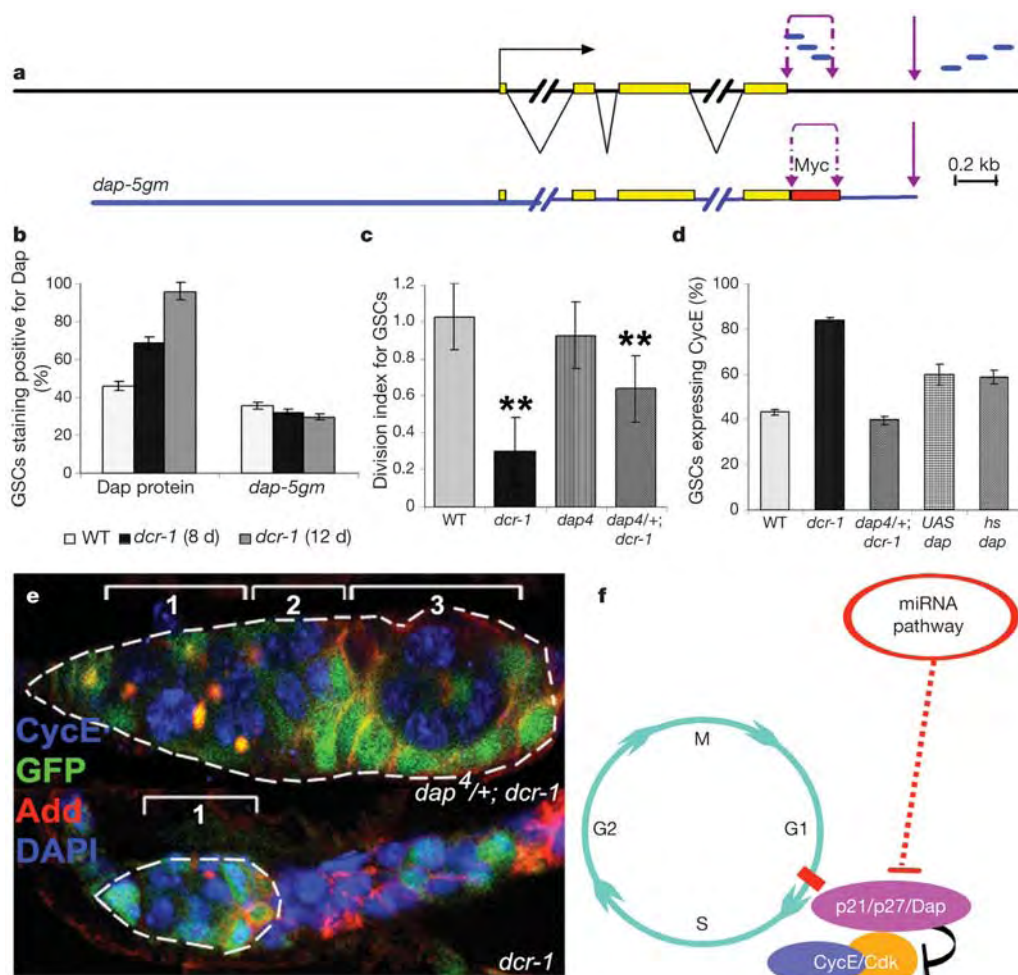


Figure 4 | The GSC division defect is dependent on Dap.

a, The *dap-5gm* transgene compared to the endogenous *dap* locus. A 6 × Myc tag replaces a region of the 3' UTR containing predicted miRNA-binding sites (blue bars). The UTR lacks the last 540 base pairs. **b**, Endogenous Dap is detected more frequently in *dcr-1* mutant than wild-type GSCs but Dap from *dap-5gm* is not detected more frequently in *dcr-1* GSCs. **c**, **e**, Germlaria with *dap4/+; dcr-1*^{Q1147X} GSCs produce more cysts than germlaria with *dcr-1*^{Q1147X} GSCs. Double asterisk, $P \leq 10^{-3}$ (Student's *t*-test). **d**, Percentage of CycE-positive GSCs. Dap was overexpressed by *nos-Gal4/pUASp-dap* or *hs-dap*. Error bars represent standard deviations (**c**) or errors from the mean (**d**). **f**, miRNA pathway modulates the GSC cell cycle by affecting the G1/S transition through Dap (p21/p27).

that expression of a Dap transgene containing the Dap promoter and essentially all of the endogenous gene except some of the 3' UTR (*dap-5gm*)²³ was similar in *dcr-1* mutant and wild-type GSCs (Fig. 4b; see also Supplementary Fig. 3b, c). These data suggest that the effect of Dcr-1 on Dap regulation in GSCs (Fig. 4b) is at a post-transcriptional level and might involve the 3' UTR region that is missing in the *dap-5gm* transgene (Fig. 4a).

We propose that miRNAs are required for GSCs to transit the G1/S checkpoint by repressing directly or indirectly the G1/S inhibitor Dap (Fig. 4f). Because Dap is a key component of the G1/S transition^{21–25}, it is a plausible target for machinery that assures continuous cell division in a microenvironment in which most of the cells are quiescent. We propose that while the TGF- β pathway—which can upregulate p21/p27 (ref. 24)—is active in GSCs¹⁶, miRNAs down-regulate Dap to assure the continuous cell division essential for stem cells. This downregulation might be direct, because the Dap 3' UTR contains several predicted miRNA-binding sites^{26–28} (Supplementary Fig. 4). A Dap transgene lacking these sites showed no response to Dcr-1 levels (Fig. 4b; see also Supplementary Fig. 3b, c), suggesting that the potential binding sites are responsive to Dicer-1. However, it is also possible that the Dap misregulation in *dcr-1* mutant GSCs might be due to a secondary effect of Dcr-1 loss. Our finding that miRNAs are required for stem cell division suggests that miRNAs might be part of a mechanism that makes stem cells insensitive to environmental signals that normally stop the cell cycle. Because miRNAs are a novel class of genes involved in human tumorigenesis²⁹, it is tempting to speculate that miRNAs could have a similar role in cancer cells.

METHODS

We used the following stocks: *eyFLP;FRT82Bdcr-1^{Q1147X}/TM3Sb*, *eyFLP;FRT82Bdcr-1^{d102}/TM3Sb*, *eyFLP;FRT42Ddcr-2^{L811X}/CyO*, *eyFLP;FRT82B parental¹⁰*, *w;FRT42Bdap⁴/CyO* (ref. 25), *dap5gm* (ref. 23), *w;NGT40/SM6a*; *nosGal4VP16/TM3Sb*, *hsFlp;FRT82Bubi-GFP/TM3Sb*, *hsFlp;FRT42Bubi-GFP/CyO*, *hsFlp;FRT42Dubi-GFP/CyO*, *FRT82BhsNmyc*.

For female germline clones, FLP-FRT flies were heat shocked (third instar larvae for 1 h, pupae for 1 h at 37 °C) and dissected 5–12 days after the last heat shock. For male germline clones, adult flies were heat shocked for 40 min at 37 °C twice per day for 3 days and dissected 2–6 days after the last heat shock.

We used the following antibodies: mouse anti-CycA, anti-CycB, anti-Adducin (1:20) and anti-Armadillo (1:40) (Developmental Studies Hybridoma Bank), mouse anti-Dap (1:5, I. Hariharan), anti-BrdU (1:20, PharMingen) and anti-c-Myc (1:50, Calbiochem), rabbit anti-PH3 (1:200, Upstate Biotechnology), anti-GFP (1:1,000, Molecular Probes), anti-Vasa (1:10,000, P. Lasko) and anti-P-Mad (1:500, P. ten Dijke), guinea-pig anti-CycE (1:500, T. Orr-Weaver), rat anti-BamC (1:1,000, D. McKearin), Alexa 488, 568, or 633 goat anti-mouse, anti-rabbit and anti-guinea-pig (1:500, Molecular Probes), and goat-anti-rat Cy5 (1:250, Jackson Immunoresearch).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Crystal structure of thymine DNA glycosylase conjugated to SUMO-1

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Members of the small ubiquitin-like modifier (SUMO) family can be covalently attached to the lysine residue of a target protein through an enzymatic pathway similar to that used in ubiquitin conjugation¹, and are involved in various cellular events that do not rely on degradative signalling via the proteasome or lysosome^{2–5}. However, little is known about the molecular mechanisms of SUMO-modification-induced protein functional transfer. During DNA mismatch repair, SUMO conjugation of the uracil/thymine DNA glycosylase TDG promotes the release of TDG from the abasic (AP) site created after base excision, and coordinates its transfer to AP endonuclease 1, which catalyses the next step in the repair pathway⁶. Here we report the crystal structure of the central region of human TDG conjugated to SUMO-1 at 2.1 Å resolution. The structure reveals a helix protruding from the protein surface, which presumably interferes with the product DNA and thus promotes the dissociation of TDG from the DNA molecule. This helix is formed by covalent and non-covalent contacts between TDG and SUMO-1. The non-covalent contacts are also essential for release from the product DNA, as verified by mutagenesis.

TDG initiates base excision repair by releasing thymine or uracil from G·T and G·U mismatches arising from the hydrolytic deamination of methyl-cytosine and cytosine bases that are paired with guanines⁶. Both deamination products can be detrimental to the cell because, unless repaired, they induce a C-to-T transition after DNA replication. After it excises the base from these mismatches, TDG remains stably bound to the resultant AP site, protecting this harmful repair intermediate until it is transferred to AP endonuclease 1 (APE1) to enable the subsequent step in the repair pathway^{7,8}. Conjugation of SUMO-1 and SUMO-2/3 to TDG markedly reduces the affinity of TDG for the AP site⁹. Therefore, SUMO conjugation probably constitutes the specific mechanism that releases TDG from the product DNA and coordinates its transfer to APE1.

Human TDG consists of a catalytic core domain (residues 123–300), which shares high sequence similarity with the *Escherichia coli* mismatch-specific uracil DNA glycosylase (MUG). The amino- and carboxy-terminal domains of human TDG are less conserved, with the C-terminal domain containing the SUMO conjugation site Lys 330, the ε-amino group of which can be linked with the C terminus of SUMO-1 via an isopeptide bond. The crystal structure shows that *E. coli* MUG binds stably to DNA by forming hydrogen bonds with the unpaired guanine residue opposite the AP site¹⁰. Mutational analyses have shown that structural features of the AP site binding are conserved between *E. coli* MUG and mammalian TDG¹¹.

To elucidate the molecular mechanism of the SUMO modification (SUMOylation)-directed release of TDG from product DNA, we have determined the crystal structure of the central region of human TDG (residues 112–339) conjugated to SUMO-1 (hereafter referred to as SUMO-1–TDG). A map of the electron densities at 2.1 Å resolution was obtained for all amino acids except for five and eighteen residues at the N terminus of TDG and SUMO-1, respectively, and the seven C-terminal residues of TDG (Fig. 1a; see also Supplementary Fig. 1). The structure shows that SUMO-1–TDG is comprised of two domains: a catalytic core domain of TDG comprising residues 117–300, and a SUMO-containing domain, consisting of the structured region of SUMO-1 and the C-terminal region (residues 307–330) of TDG (Fig. 1b). These domains are connected by a short crossover loop comprising residues 301–306 of TDG.

The catalytic core domain, but not the C-terminal segment, of TDG does not seem to undergo substantial structural rearrangements upon conjugation. The structure of the TDG core domain closely resembles those of mismatch DNA glycosylases, such as MUG from *E. coli* and mammalian uracil DNA glycosylases (UDGs). The root-mean-square deviation (r.m.s.d.) of 122 Cα atoms between the TDG core domain and *E. coli* MUG (Protein Data Bank (PDB) code 1MWI) is 1.4 Å. The alignment of these structures superimposes the pyrimidine-binding pocket of MUG onto a pocket of TDG, suggesting that the pocket of the catalytic core of TDG is also involved in the nucleotide flipping mechanism commonly used in UDG enzymes^{10,12–14} (Fig. 1b). Residues that are important for DNA binding and the catalytic activity of MUG are also effectively superimposed onto the TDG core domain, indicating high structural conservation throughout evolution¹¹. These observations also suggest that no major structural rearrangement of the TDG core domain occurs upon SUMO-1 conjugation. Furthermore, SUMO-1 does not undergo substantial conformational changes after conjugation, because the overall fold of the structured region of SUMO-1 (residues 19–97) in the SUMO-1–TDG complex is nearly identical to that in unconjugated SUMO-1 (PDB code 1A5R), as shown by a r.m.s.d. of the Cα atoms of 2.2 Å. The 18 N-terminal amino acids for which electron densities are not observed in the SUMO-1–TDG crystal have been shown to be flexible in unconjugated SUMO-1 in solution¹⁵.

The major molecular interface between TDG and SUMO-1 is formed by the C-terminal segment (residues 307–330) of TDG. The elongated structure of this segment wraps around SUMO-1, forming the globular SUMO-containing domain (Fig. 1a, b). The segment

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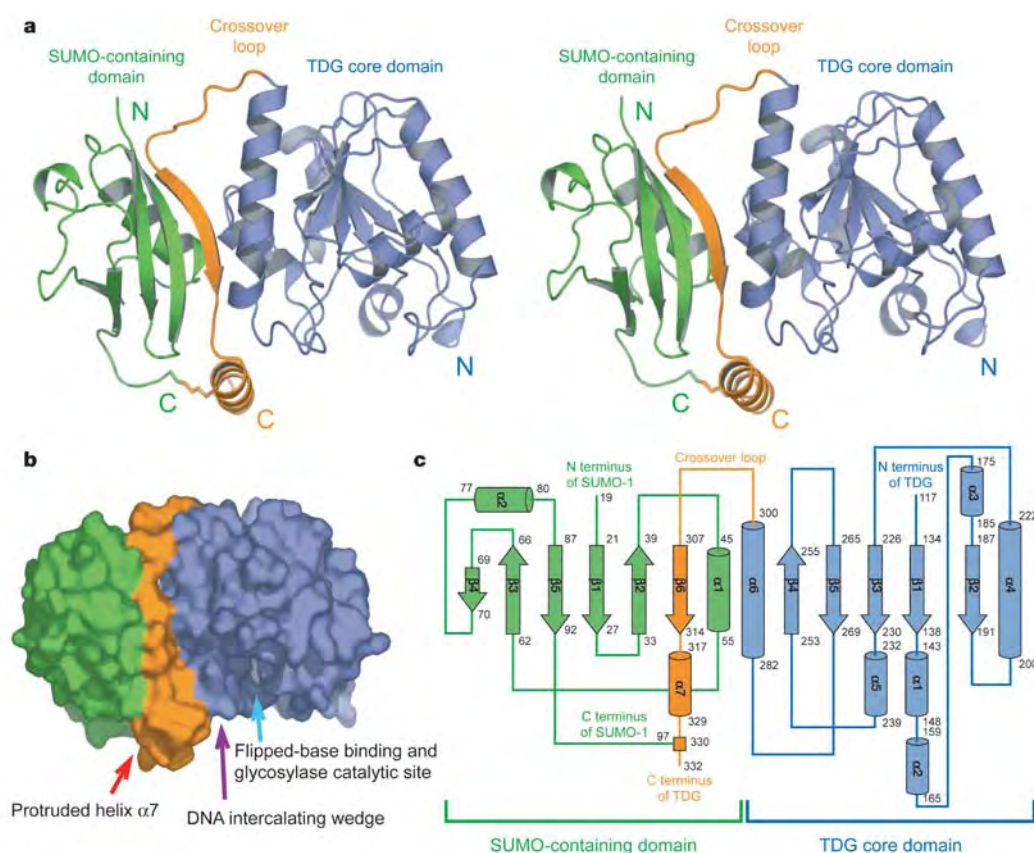


Figure 1 | Structure of SUMO-1-TDG. **a**, Stereo ribbon diagram of the structure of SUMO-1-TDG. The catalytic core domain and the C-terminal segment of TDG are shown in purple and orange, respectively; SUMO-1 is shown in green. **b**, Surface of SUMO-1-TDG. The colouring and viewing angle are the same as in **a**. The positions of the protruded helix $\alpha 7$, putative base-binding pocket and DNA intercalating wedge are also shown. **c**, Topology of the secondary structure elements. Residue numbers and the N and C termini of TDG and SUMO-1 are indicated. The colouring is the same as in **a** and **b**.

makes both covalent and non-covalent contacts with SUMO-1. The covalent contact occurs at the C terminus of this segment through the isopeptide bond, and the non-covalent contacts occur at strand $\beta 6$ (residues 307–314) at the N terminus of the segment (Fig. 1c). This strand forms an intermolecular antiparallel β -sheet with strand $\beta 2$, an edge strand of the β -sheet of SUMO-1, resulting in a continuous six-stranded, mixed β -sheet. In addition to their main chain contacts, the side chains of residues from TDG $\beta 6$ make extensive polar and hydrophobic contacts with those from $\beta 2$ and $\beta 1$ of SUMO-1

(Fig. 2a, b). Glu 310 of TDG makes bidentate hydrogen bonds to Arg 54 in SUMO-1, whereas the side chain of Val 308 packs into a pocket formed by the side chains of Phe 36, Val 38 and Leu 47 of SUMO-1. The intermolecular contacts also involve a hydrogen bond network formed between Arg 281, Asp 284, Tyr 313 and Asp 323 of TDG and Asp 30 of SUMO-1. The interface between SUMO-1 and TDG is largely confined to the edge of the β -sheet of SUMO-1.

The most prominent feature of SUMO-1-TDG is helix $\alpha 7$ of TDG, which contains the conjugation site. Buttressed by this conjugation

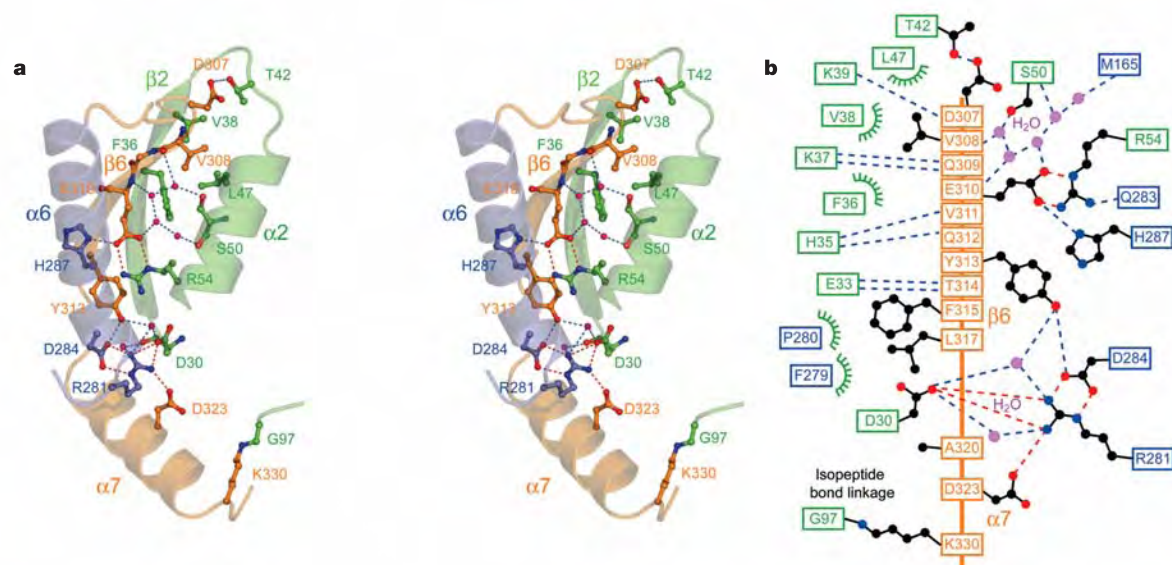


Figure 2 | Molecular interface between TDG and SUMO-1. **a**, Detailed stereo view of contacts between TDG and SUMO-1. Residues from the C-terminal segment and core of TDG are shown in orange and purple, respectively; residues from SUMO-1 are shown in green. **b**, Schematic

summary of contacts in SUMO-1-TDG. Hydrophobic, hydrogen bond and electrostatic interactions are shown in green, blue and red, respectively. Protein residues are coloured as in **a**.

site at its C terminus and the intermolecular β -sheet pairing of strand $\beta 6$ flanking its N terminus, this helix is held up above SUMO-1 and forms a large protrusion on the protein surface (Fig. 1b). In particular, the C-terminal end of the helix makes almost no non-covalent contacts with other parts of the proteins, and thus is raised only by the isopeptide bond between Lys 330 and SUMO-1.

The strong conservation of tertiary structures and residues that are important for DNA binding and catalysis between the catalytic core domain of TDG and *E. coli* MUG suggests that these enzymes share a similar mode of DNA interaction and catalytic mechanism. We therefore constructed a model of the complex formed between TDG and product DNA by best-fit superposition of the coordinates of the TDG core domain to those of MUG bound to a product DNA containing an AP site¹⁰ (see Methods). Notably, $\alpha 7$, the protruded helix at the C-terminal segment of TDG, is positioned such that it would encounter severe steric clash with the sugar-phosphate backbone of the bound DNA at positions +1 and +2 from the unpaired guanine in the TDG–DNA model (Fig. 3). Furthermore, it seems unlikely that the bound DNA could undergo a bend or deformation to avoid this apparent steric clash, because the model suggests that loops between strand $\beta 5$ and helix $\alpha 6$, and between helices $\alpha 1$ and $\alpha 2$, make contact with the sugar-phosphate backbone of the AP-site-containing strand at positions 0 and –1, in very close proximity to the putative clash site. Therefore, it seems most likely that this steric clash between the protruded helix and the DNA backbone induces the dissociation of SUMO-1–TDG from the AP site on DNA. It is noteworthy that SUMO-1 is positioned so that it does not encounter steric interference from any part of the DNA in the model.

Structural features of the complex suggest that maintenance of $\alpha 7$,

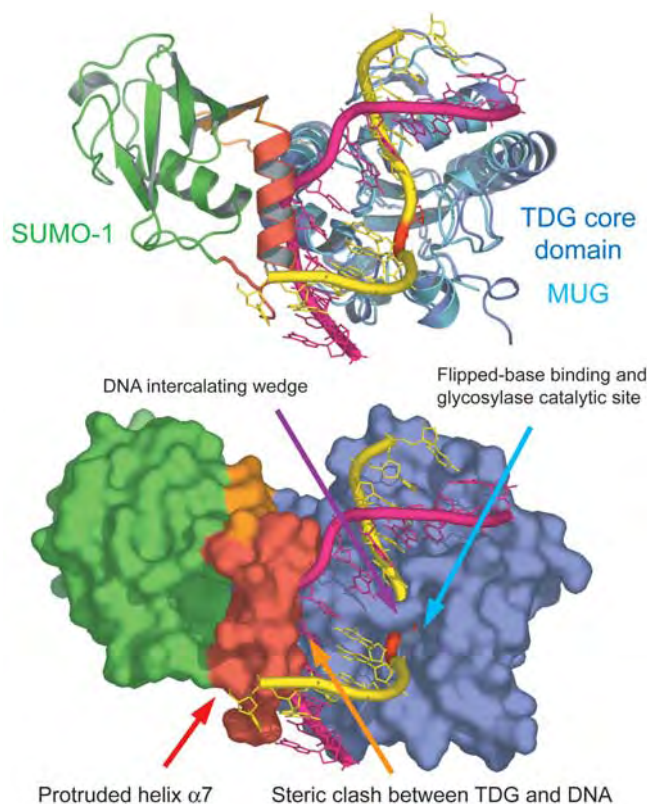


Figure 3 | Model of a complex between SUMO-1–TDG and a DNA molecule containing an AP site. The model was constructed by superimposing the structure of SUMO-1–TDG on that of MUG bound to a product DNA containing an AP site¹⁰ (PDB code 1MWI). A canonical B-form conformation outside the AP sites has been assumed. The locations of the putative base-binding pocket and DNA intercalating wedge are indicated. The ‘protruded helix’ $\alpha 7$ is shown in red, and the site of putative steric clash between the helix and DNA backbone is also indicated.

the protruded helix, requires both SUMO-1 conjugation and β -sheet pairing between $\beta 6$ of TDG and $\beta 2$ of SUMO-1 (Fig. 2). Except for these interactions, the helix makes only a few contacts with other parts of the proteins. Therefore, the residues that form this helix are probably unstructured when SUMO-1 is not conjugated. Consistent with this, unconjugated TDG is more sensitive to the proteases trypsin and thermolysin than is its SUMO-1-conjugated form (Supplementary Fig. 2). Therefore, the formation of this protruded helix is most probably the consequence of a structural rearrangement of the C-terminal segment of TDG, induced by SUMO-1 conjugation.

We carried out mutational analyses that showed that the non-covalent interactions between the C-terminal segment of TDG and SUMO-1 are indispensable for releasing SUMO-1–TDG from the product DNA. Glutamine substitution for Glu 310 of TDG, which makes bidentate hydrogen bonds with Arg 54 of SUMO-1, largely restored the ability of SUMO-1–TDG to bind to the product DNA (Fig. 4a). Similarly, substitution of Arg 281 of TDG—which is involved in the hydrogen bond network formed between TDG and SUMO-1—by alanine recovered the DNA binding of SUMO-1–TDG. Notably, these residues are essential for the previously observed⁹ non-covalent binding of TDG to SUMO-1, as shown by glutathione S-transferase (GST) pull-down assays (Fig. 4b). Phe 315 is another residue for which alanine substitution resulted in a loss of SUMO-1 binding and recovery of the DNA binding of SUMO-1–TDG (Fig. 4a, b). Therefore, both the non-covalent and covalent interactions that fix the C-terminal segment of TDG seem to be essential for the release of DNA from SUMO-1–TDG.

Notably, strand $\beta 6$, the major intermolecular contact region of TDG, contains a sequence (VQEV, residues 308–311; Fig. 2b) that is not identical, but is similar, to the recently proposed SUMO-binding motif (SBM)¹⁶. The SBM has the consensus sequence V/I-X-V/I-V/I and is found in proteins that bind to SUMO family proteins. Mutagenesis has confirmed that residues in the SBM-like sequence are important for non-covalent SUMO-1 binding (Fig. 4b). These observations suggest that the SBM found in other proteins may bind

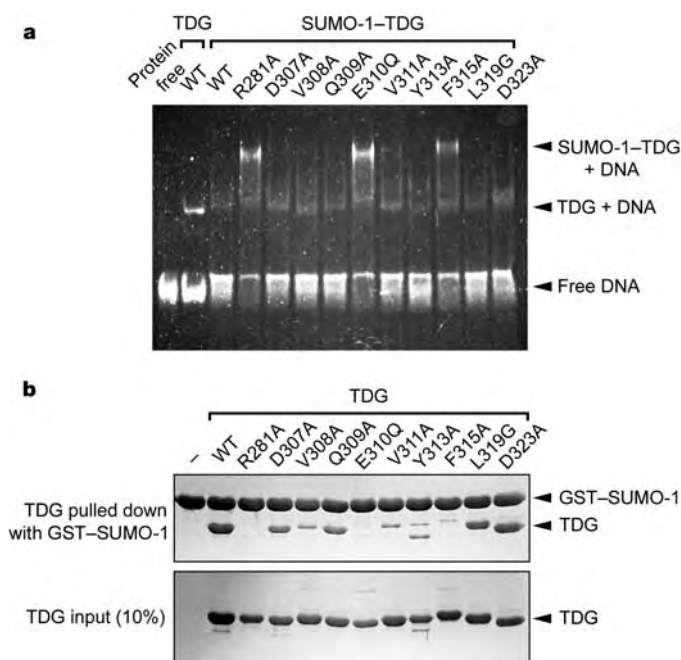


Figure 4 | DNA and SUMO-1 binding activity of TDG. **a**, Electrophoretic mobility shift assay examining the DNA-binding capacity of different TDG mutants conjugated to SUMO-1. **b**, GST pull-down assay examining the SUMO-1 binding capacity of different mutants of unconjugated TDG. The concentration of TDG and its mutants as well as SUMO-1 was 10 μ M.

to SUMO family proteins through the formation of an intermolecular β -sheet, similar to that seen in SUMO-1-TDG. Consistent with this, it has been shown that residues from strand $\beta 2$ of SUMO-1 display marked perturbations in chemical shifts upon binding to an SBM-containing peptide¹⁶. Of note, many SUMOylated proteins contain an SBM consensus sequence¹⁶. This raises the possibility that protein segments between the SBM and the SUMOylation site of these proteins may undergo a conformational rearrangement similar to that seen in SUMO-1-TDG. In conclusion, the structure of SUMO-1-TDG suggests that SUMO-1 conjugation induces the formation of the protruded helix in TDG, which allows its dissociation from the product AP site. It cannot be ruled out that formation of the helix may also induce larger conformational changes in the enzyme that facilitate its functional transfer.

METHODS

Crystallization and structure determination. Proteins are expressed and purified as described¹⁷ (see also Supplementary Methods). Crystals of SUMO-1-TDG were grown in 25% PEG3350, 0.2 M MgCl₂ and 0.1 M Tris-HCl (pH 8.5) at 20 °C by using a micro-seeding technique. The crystals belong to space group $P2_12_12_1$ with unit-cell dimensions of $a = 42.2 \text{ \AA}$, $b = 70.4 \text{ \AA}$ and $c = 106.4 \text{ \AA}$. X-ray diffraction data were collected at beamline BL6A of the Photon Factory (Tsukuba, Japan) with a Quantum R4 CCD detector (ADSC) at wavelength 1.0 Å. The diffraction data were processed with MOSFLM¹⁸ and scaled with SCALA¹⁹. The 2.1 Å resolution structure of SUMO-1-TDG was solved by molecular replacement with MolRep²⁰ using *E. coli* MUG¹⁰ (PDB code 1MUG) and yeast Smt3 (ref. 21) (PDB code 1EUV) as search models. The model building was done with program O²² and refined with CNS²³. The Ramachandran plot of the final model shows that 93.8% of the residues are in the most favoured regions, and 6.2% in additionally favoured regions. The current model gives R_{work} and R_{free} values of 20.5% and 24.5%, respectively. The data collection and refinement statistics are summarized in Supplementary Table 1. Figures 1a, b, 2a and 3 were made with the program PyMOL²⁴.

Model building of a SUMO-1-TDG-DNA complex. The model of a complex between SUMO-1-TDG and a DNA molecule containing an AP site was constructed by superimposing the structure of SUMO-1-TDG on that of MUG bound to a product DNA containing an AP site¹⁰ (PDB code 1MWI).

We assumed that the DNA adopts a canonical B-form conformation, because DNA molecules in MUG complexes have been supposed to maintain B-form conformations outside the AP sites^{10,25}. We found that most of the protein residues that make DNA contacts and form the pyrimidine-binding pocket are located at similar positions relative to the DNA in the MUG-DNA complex and in the model, supporting the validity of our model.

Biochemical assays. A fluorescein isothiocyanate (FITC)-labelled 35-mer double-stranded oligonucleotide substrate containing a U-G mismatch (5'-GGCAATCAGTTCAGTTCGAGCCAGGTATTAGCC-3'; 5'-FITC-GGCTAAATACCTGGGCTUGAAGTGAAGTATTGCC-3') was chemically synthesized and annealed in a buffer containing 10 mM Tris-HCl, 5 mM MgCl₂ and 0.1 mM EDTA (pH 7.5). An oligonucleotide containing an AP site was generated by incubating 10 pmol of the duplex containing the U-G base pair with 1 unit of uracil DNA glycosylase (New England Biolabs) in a buffer containing 20 mM Tris-HCl, 1 mM EDTA and 1 mM dithiothreitol (DTT) (pH 8.0) for 2 h at 37 °C. The accuracy and completion of AP site formation were analysed by NaOH treatment and denaturing gel electrophoresis. For the electrophoretic mobility shift assay, 4 pmol of TDG proteins were incubated in 10 μ l of reaction mixture (25 mM HEPES-NaOH, 150 mM NaCl, 5 mM MgCl₂, 0.01% Triton X-100, 1 mM EDTA, 5% glycerol, 1 mM DTT and 0.1 mg ml⁻¹ BSA; pH 7.5) with 1 pmol of the oligonucleotide duplex containing an AP site and 5 pmol of the non-labelled homoduplex competitor. After incubation for 30 min at 30 °C, the samples were immediately loaded onto 8% native polyacrylamide gels in 0.5 $\times$ TBE at 100 V at room temperature. The fluorescent probes were visualized by using a luminescent image analyser LAS-1000 Plus (Fuji Photo Film) in the fluorescence mode.

The non-covalent interactions between SUMO-1 and TDG were analysed by GST pull-down experiments. Purified GST-SUMO-1(1-97) was immobilized onto glutathione-sepharose beads. Various TDG mutants were added to the beads and incubated at 4 °C for 1 h in a buffer containing 25 mM HEPES, 150 mM NaCl, 1 mM DTT and 0.05% NP-40 (pH 7.5). The beads were then washed three times with the same buffer and analysed by SDS-polyacrylamide gel electrophoresis with Coomassie brilliant blue stain. The standard condition

used 50 μ l of the beads and 100 μ l of the GST fusion protein and various TDG mutants (10 μ M).

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information Atomic coordinates of SUMO-1-TDG have been deposited in the Protein Data Bank under the accession number 1WYV. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to M.S. (shirakawa@moleng.kyoto-u.ac.jp).

Structural basis for the promiscuous biosynthetic prenylation of aromatic natural products

Tomohisa Kuzuyama^{1,2}, Joseph P. Noel¹ & Stéphane B. Richard¹

The anti-oxidant naphterpin is a natural product containing a polyketide-based aromatic core with an attached 10-carbon geranyl group derived from isoprenoid (terpene) metabolism^{1–3}. Hybrid natural products such as naphterpin that contain 5-carbon (dimethylallyl), 10-carbon (geranyl) or 15-carbon (farnesyl) isoprenoid chains possess biological activities distinct from their non-prenylated aromatic precursors⁴. These hybrid natural products represent new anti-microbial, anti-oxidant, anti-inflammatory, anti-viral and anti-cancer compounds. A small number of aromatic prenyltransferases (PTases) responsible for prenyl group attachment have only recently been isolated and characterized^{5,6}. Here we report the gene identification, biochemical characterization and high-resolution X-ray crystal structures of an architecturally novel aromatic PTase, Orf2 from *Streptomyces* sp. strain CL190, with substrates and substrate analogues bound. *In vivo*, Orf2 attaches a geranyl group to a 1,3,6,8-tetrahydroxynaphthalene-derived polyketide during naphterpin biosynthesis. *In vitro*, Orf2 catalyses carbon–carbon-based and carbon–oxygen-based prenylation of a diverse collection of hydroxyl-containing aromatic acceptors of synthetic, microbial and plant origin. These crystal structures, coupled with *in vitro* assays, provide a basis for understanding and potentially manipulating the regio-specific prenylation of aromatic small molecules using this structurally unique family of aromatic PTases.

Naphterpin is produced via both mevalonate (MVA) and polyketide biosynthetic pathways. Naphterpin biosynthesis includes

1,3,6,8-tetrahydroxynaphthalene (THN), which after oxidative transformation to flaviolin, is prenylated with a 10-carbon geranyl moiety before final biosynthetic elaboration of the product³ (Fig. 1). To understand the biosynthesis of this hybrid isoprenoid-polyketide-derived natural product, the gene cluster responsible for naphterpin production was identified based upon proximity to genes encoding the MVA pathway (T. Kumano *et al.*, unpublished data)

An upstream region of the gene cluster containing the MVA pathway genes revealed three new open reading frames (ORFs) designated *orf1*, *orf2* and *orf3* (Supplementary Table S1). PSI-BLAST searches revealed strong homologies between Orf2 and three other bacterial proteins: a hypothetical protein from *Streptomyces coelicolor* A3(2) (HypSc, GenBank accession number AL939130) and the previously described 4-hydroxyphenylpyruvate: dimethylallyl transferase proteins encoded by the genes *cloQ* (accession number AF329398) and *novQ* (accession number AF170880) from *Streptomyces roseochromogenes* and *Streptomyces spheroides* NCIMB 11891, respectively⁵ (Supplementary Fig. S1). A recently identified aromatic PTase from *Lyngbya majuscula* (accession number AY588942) involved in lyngbyatoxin biosynthesis displays low similarity with Orf2⁶. The *Streptomyces* sp. CL190 mutant strain resulting from disruption of the *orf2* gene exhibited no naphterpin production. Moreover, the high degree of homology between Orf2 and the functionally characterized PTases CloQ and NovQ⁵, and the fact that Orf3 is a type III polyketide synthase with amino acid similarity to THN synthase (Supplementary Table S1)^{7,8}, strongly

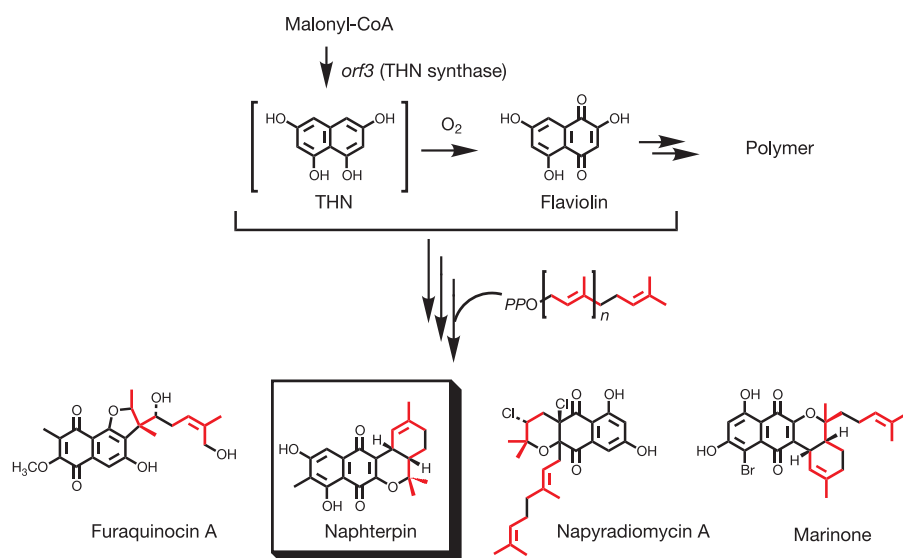


Figure 1 | Biosynthesis of naphterpin and other hybrid isoprenoid-polyketide compounds produced by Actinomycetes. The synthesis of naphterpin involves the prenylation of THN, flaviolin or a derived metabolite using a GPP co-substrate. The THN skeleton is further modified, prenylated and incorporated into hybrid isoprenoid-polyketides such as naphterpin, furaquinocin A, napyradiomycin A and marinone. Each isoprenoid C5 unit is shown in red.

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suggest that *orf2* encodes a PTase involved in geranyl group transfer to THN or a THN metabolite resulting from additional biosynthetic elaborations catalysed by Orf3 and possibly other tailoring enzymes.

When expressed in *Escherichia coli*, Orf2 behaves as a soluble 33-kDa (307 residues) monomeric protein. To assess enzyme activity, purified recombinant Orf2 was incubated with putative aromatic acceptors in the presence of dimethylallyl diphosphate (DMAPP, C5), geranyl diphosphate (GPP, C10) or farnesyl diphosphate (FPP, C15). The resultant PTase activity of Orf2 displayed relaxed substrate specificity for aromatic small molecules, as prenylated products accumulate using THN analogues including 1,3-dihydroxynaphthalene (1,3-DHN), 1,6-DHN and 2,7-DHN, as well as flavioline (Fig. 2a, b). Orf2 also catalyses the prenylation of the CloQ substrate 4-hydroxyphenylpyruvate (4-HPP) (Fig. 2b). For the isoprenoid diphosphate substrates, Orf2 exhibited no activity with DMAPP, the highest relative activity with GPP and detectable activity with FPP.

Although the true physiological substrate of Orf2 is still under investigation, significant Mg^{2+} -dependent, *in vitro* activity is observed with the dihydroxy-containing THN analogues (Fig. 2a, b). Notably, the formation of two prenylated products, 1,6-DHN-P1 and 1,6-DHN-P2, was readily detected by thin-layer chromatography (TLC) when Orf2 was incubated with both 1,6-DHN and GPP. Large-scale incubations with GPP and 1,6-DHN produced a sufficient amount of both products—in a 10:1 ratio—to permit their structural elucidation using both mass spectrometry (MS) and 1H nuclear magnetic resonance (NMR) analyses (see Supplementary Data). Each compound possessed a single geranyl chain at C2 and C5,

resulting in *trans*-2-geranyl 1,6-DHN and *trans*-5-geranyl 1,6-DHN, respectively (Fig. 2a).

Orf2 was next assayed against various flavonoids, isoflavonoids and related plant polyketides including resveratrol, olivetol and olivetolic acid, for which significant activity was observed (Fig. 2b). In the presence of naringenin (5,7,4'-trihydroxyflavanone) and GPP, two reaction products, 6-geranyl naringenin and 7-O-geranyl naringenin, form (Fig. 2b). 6-geranyl naringenin, also known as bonannione A⁹, is a prenylated flavanone displaying significant antibacterial activity¹⁰. 7-O-geranyl naringenin is, to the best of our knowledge, a novel prenylated flavonoid. Orf2 also geranylated olivetol and olivetolic acid (Fig. 2b). These two plant polyketides and their geranylated products serve as intermediates in the biosynthesis of the plant-derived polyketide-terpene natural product Δ^9 -tetrahydrocannabinol (Δ^9 -THC)¹¹.

To investigate the architectural principles underlying prenyl chain-length determination, aromatic substrate selectivity and the mechanism of prenyl group transfer, we determined four X-ray crystal structures of Orf2 substrate/substrate analogue complexes, including Orf2 bound to a TAPS buffer molecule, a binary Orf2 complex containing GPP and Mg^{2+} , a ternary Orf2 complex containing a non-hydrolysable GPP analogue, geranyl *S*-thiolodiphosphate (GSPP), Mg^{2+} and 1,6-DHN, and a ternary Orf2 complex containing GSPP, Mg^{2+} and flavioline (Supplementary Table S2). The three-dimensional structure of Orf2 consists of a single domain possessing a novel barrel fold (Fig. 3). This new barrel, here termed a PT barrel, is a cylindrical β -sheet comprising ten anti-parallel β -strands arranged around a central solvent-filled core. In an arrangement reminiscent of a TIM barrel, the cylindrical β -sheet is surrounded by

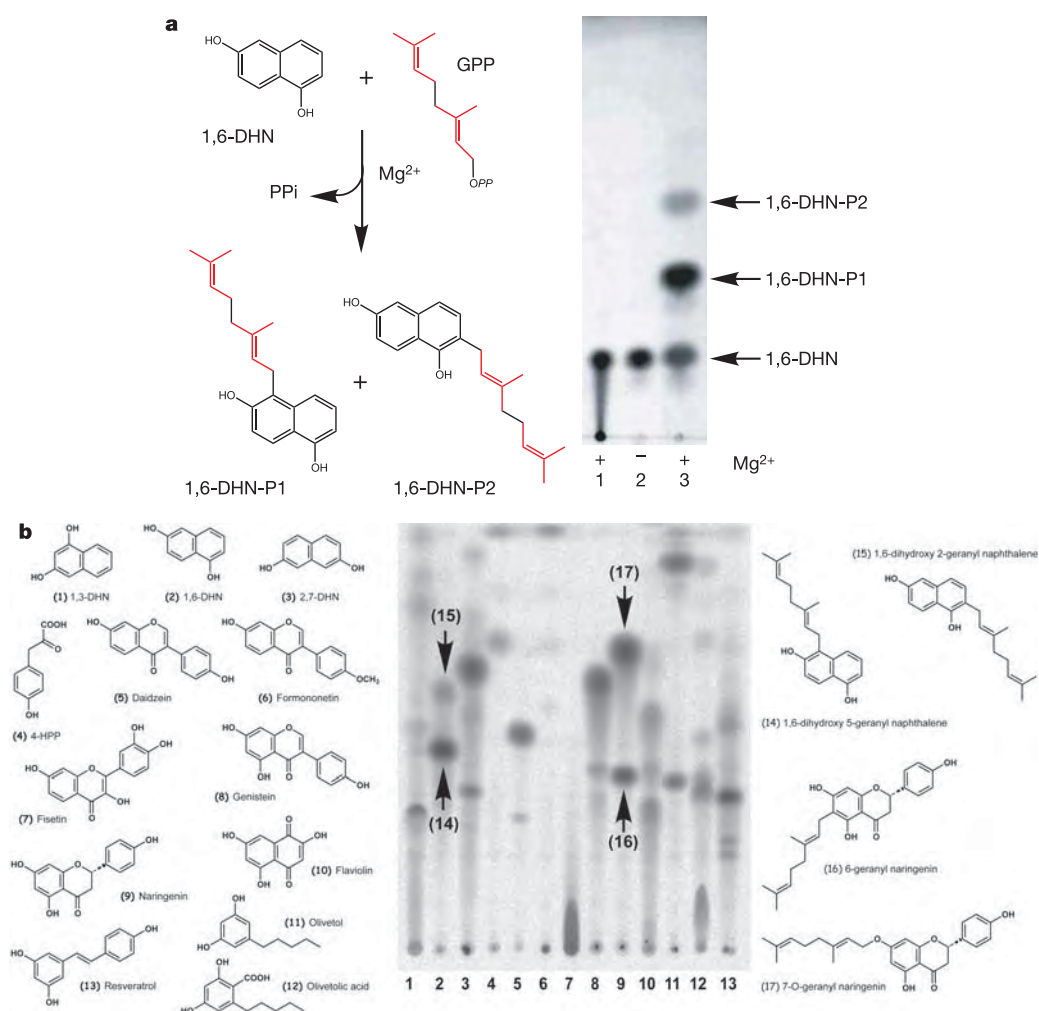


Figure 2 | Enzymatic assays of Orf2. **a**, Mg^{2+} -dependent prenylation of 1,6-DHN. In lane 1 (control), Orf2 was boiled before addition. The reaction mixture analysed in lane 2 contained no $MgCl_2$, whereas 5 mM $MgCl_2$ was added in lane 3. **b**, Promiscuous activity against chemically diverse aromatic acceptors. Assays used the substrates named and numbered on the left side of the TLC. The chemical structures of four reaction products were determined by MS/NMR analyses and are shown to the right of the TLC.

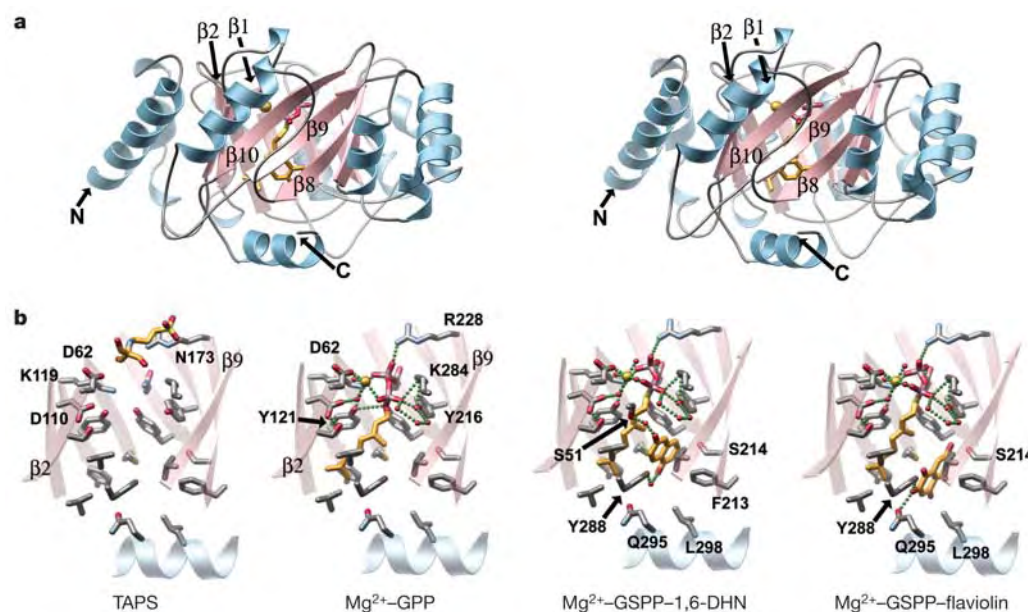


Figure 3 | Structures of Orf2's PT barrel. **a**, Stereo view of the Orf2 monomer viewed with the central barrel axis oriented vertically in the plane of the page. The cylindrical solvent-filled β -sheet and outer belt of α -helices are differentially coloured. Bound GSPP and 1,6-DHN residue within the PT barrel and are represented as colour-coded sticks. Mg^{2+} is shown as a gold-coloured van der Waal's sphere. **b**, Orf2 substrate/substrate analogue complexes. Only the β -strands (minus β -strands 1 and 10) plus the C-terminal α -helix are displayed in the same vertical orientation as in **a** after a 30° anticlockwise rotation around the barrel axis. Hydrogen and coordination bonds are shown as green spheres.

a ring of solvent-exposed α -helices; however, the connectivity of Orf2's secondary structure elements is not shared with TIM barrels (Supplementary Fig. S2b).

The secondary connectivity nearly conforms to a $(\alpha\alpha\beta\beta)_5$ classification, but is more specifically described using the $(\alpha\alpha\beta\beta)_4$ -($\alpha\beta\beta$)- α nomenclature due to a helical kink between α -helices 7 and 8. Searches for structurally related proteins retrieved examples belonging to either the TIM barrel or the β -barrel structural families (Supplementary Fig. S2), both of which display barrel folds with connectivity patterns that are distinctively different from the PT barrel described here^{12–15} (see Supplementary Discussion). A number

of hydrophobic residues located inside the PT barrel sequester the geranyl tail of GPP and GSPP, whereas the diphosphate or the thio-diphosphate head groups, respectively, point towards the 'upper', more polar end of the barrel where a Mg^{2+} ion is coordinated.

Whereas Orf2 and its structural homologue CloQ display considerable aromatic PTase activities, the primary sequence and tertiary structure of Orf2 are not shared with any known PTase or mechanistically related biosynthetic enzymes^{16–22}. Moreover, the sequence does not contain the (N/D)DXXD signature motif indicative of Mg^{2+} -dependent isoprenoid diphosphate recognition. Even within the Orf2 family of PTases, some members such as CloQ exhibit

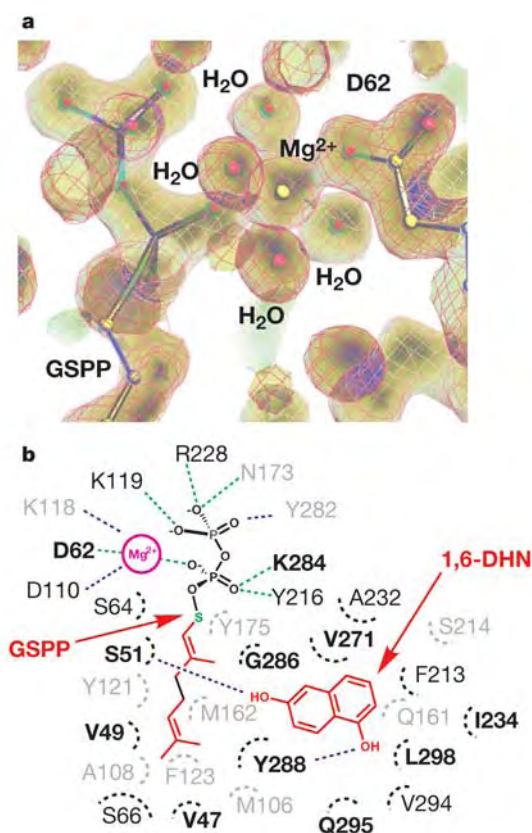


Figure 4 | Mg^{2+} dependence and substrate recognition in aromatic PTases. **a**,

Representative $2F_o - F_c$ electron density map, contoured at 1.0σ , displaying the octahedral coordination of the catalytic Mg^{2+} . The orientation is the same as that in Figs 3b and 4b after a 180° rotation around the vertical barrel axis. **b**, Schematic representation of Orf2's active site. The side chains involved in Mg^{2+} , GSPP and 1,6-DHN binding are depicted with direct hydrogen and coordination bonds shown as green dashes and water-mediated bonds as blue dashes. Half circles depict van der Waal's contacts with grey for residues in the back of the GSPP-1,6-DHN plane, black in the same plane and thick black in the front. **c**, Biochemical characterization of HypSc. PTase activity was assayed and visualized as in Fig. 2a using 1,6-DHN as a prenyl acceptor and either DMAPP or GPP as potential prenyl donors.

Product →				
1,6-DHN →				
Mg^{2+}	+	-	+	+
DMAPP	-	+	+	-
GPP	-	-	-	+
HypSc	+	+	+	+
	1	2	3	4

activity in the absence of Mg^{2+} ions⁵, whereas Orf2's PTase activity is Mg^{2+} dependent (Fig. 2a). Complexes with Mg^{2+} and GPP, or a non-hydrolysable analogue, GSPP, precisely define the binding of GPP and the coordination of Mg^{2+} (Fig. 3b). Lys 119, Asn 173 and Arg 228, located near the polar open end of the barrel, tether the β -phosphate of GSPP (Fig. 4a). The α -phosphate linked to the geranyl chain forms a hydrogen bond with Tyr 216 and Lys 284, and also coordinates a Mg^{2+} ion. The bound Mg^{2+} exhibits octahedral coordination, with four equatorially arranged water molecules and two axially located oxygen atoms contributed by the side chain carboxylate of Asp 62 and a non-bridging oxygen of the aforementioned α -phosphate (Fig. 4a, b). Tyr 121 resides within hydrogen-bonding distance of the bridging atom (sulphur in GSPP and oxygen in GPP) linking the diphosphate moiety to the C10 geranyl chain. Finally, the hydrophobic geranyl chains of the GPP or GSPP molecules rest against the side chains of Val 49, Phe 123, Met 162, Tyr 175 and Tyr 216 (Fig. 4b).

The ternary complexes with Mg^{2+} , GSPP and either 1,6-DHN or flaviolin delineate the surprisingly spacious aromatic substrate binding pocket (Figs 3b and 4b), partially explaining Orf2's relaxed specificity for aromatic small-molecule substrates. 1,6-DHN rests against the geranyl tail of GSPP, and the bicyclic aromatic rings are sequestered between the side chains of Met 162 and Phe 213. The side chains of Gln 295 and Leu 298, provided by the short carboxy-terminal helical cap of the PT barrel, line the wall of the aromatic substrate binding pocket, with additional contacts made through the side chains of Phe 213, Ser 214 and Tyr 288 (Fig. 3b). Although the aromatic planes of both 1,6-DHN and flaviolin reside in the same active site orientation, the flaviolin molecule binds in a slightly different position than 1,6-DHN, with extra pairs of hydrogen bonds formed with Ser 214, Tyr 288 and Gln 295 (Fig. 3b).

At least two distinct catalytic mechanisms can be considered for prenylation of aromatic substrates. One reaction invokes carbon-mediated nucleophilic attack on C1 of GPP, with the diphosphate moiety stabilized by Mg^{2+} coordination and the basic character of the diphosphate binding site serving as a leaving group. This SN2-like mechanism has been described for protein farnesyltransferase (PFTase)¹⁷. A second mechanism invokes carbocation-mediated electrophilic capture, as proposed for the *trans*-prenyltransferase reaction of FPP synthase²¹ and numerous terpene synthases (cyclases) of secondary metabolism²² (see Supplementary Discussion). The distance between the site of C-prenylation on 1,6-DHN and C1 of GSPP is 4 Å. Because C1 of GPP is spatially constrained by covalent attachment to the electrostatically coordinated diphosphate moiety, PTase-catalysed cleavage of the carbon–oxygen bond before prenylation of the aromatic acceptor is more consistent with an electrophilic aromatic substitution reaction in Orf2 (Supplementary Fig. S3). Ongoing kinetic studies using 2-fluoro-substituted GPP (2F-GPP) will provide an experimental means to ascertain the most likely mechanism.

In order to decipher prenyl diphosphate chain-length selectivity, aromatic substrate recognition and divalent cation dependence, homology modelling of CloQ, NovQ and HypSc was carried out using Orf2 as a structural template (Supplementary Fig. S4). Asp 62, involved in the diphosphate binding site of Orf2 via a coordinated Mg^{2+} , is replaced in HypSc by Asn 63 and in CloQ and NovQ by Ser 64. As a possible explanation for the Mg^{2+} -independent PTase activity of CloQ and NovQ, Ser 51 in Orf2 is replaced by Lys 54 in CloQ and NovQ, and Arg 51 in HypSc. These basic residues can be positioned over the Mg^{2+} observed in the Orf2 active site. These notable active site differences in CloQ/NovQ and HypSc possibly serve as catalytic surrogates for Mg^{2+} -mediated α -phosphate binding and catalysis, supporting the Mg^{2+} -independent activity previously observed for CloQ and NovQ (ref. 5), and suggestive of a Mg^{2+} -independent mechanism in HypSc. Also, in both the CloQ/NovQ and HypSc models, residues equivalent to Ser 64 and Gly 286 in Orf2 are replaced by Arg and Glu residues, respectively

(Supplementary Fig. S4). The presence of putative salt bridges across these two positions in the prenyl diphosphate binding sites of CloQ, NovQ and HypSc appear to prevent the binding of a prenyl donor with an isoprenoid chain longer than dimethylallyl, again supporting the observed selectivity of CloQ and NovQ for the five-carbon DMAPP substrate⁵.

These models not only explain the chain-length selectivity and Mg^{2+} -independent catalytic activity measured for CloQ and NovQ but suggest that HypSc should exhibit DMAPP specificity and Mg^{2+} -independent PTase activity as well. To test these hypotheses, HypSc was overexpressed in *E. coli* as an octahistidine-tagged protein and assayed either in the absence or presence of $MgCl_2$ using DMAPP and GPP as prenyl donors and 1,6-DHN as an aromatic acceptor. Measurable PTase activity was detected when using DMAPP and 1,6-DHN as substrates in the absence or presence of Mg^{2+} , supporting the chain-length selectivity and Mg^{2+} -independent catalytic activity of HypSc first suggested from its homology model described above (Fig. 4c).

The present work describes the identification of two novel aromatic prenyltransferases: Orf2 from *Streptomyces* sp. CL190 and HypSc from *Streptomyces coelicolor* A3(2), each possessing PTase activity against aromatic small molecule substrates. Owing to Orf2's relaxed specificity for aromatic substrates, it can be used as an efficient biological catalyst for the regio-specific prenylation of aromatic small molecules. Moreover, the experimental crystal structures of Orf2 and the homology models of phylogenetically related PTases serve as starting points for rationally engineering the *in vitro* and *in vivo* prenylation of natural products of both microbial and plant origin.

METHODS

Protein expression and purification. The *orf2* gene from *Streptomyces* sp. strain CL190 (GenBank accession AB187169) was amplified by polymerase chain reaction (PCR) from total genomic DNA using oligonucleotides designed for ligation into the *E. coli* expression vector pQE30 (Qiagen), generating pQEORF2. PCR amplification using pQEORF2 and oligonucleotides for ligation into the *E. coli* expression vector pHis8 (ref. 23) was carried out with the forward primer 5'-GGGGGGGATCTCCGAAGCCGCTGATGTCG-3' (*Bam*HI site underlined) and the reverse primer 5'-GGGGGGGAATTCTCAGTCCTCCAGC GAGTCG-3' (*Eco*RI site underlined) to generate pHis8ORF2. Constructs of pHis8ORF2 were transformed into *E. coli* BL21 (DE3). Recombinant Orf2 protein was obtained and purified using a protocol previously described²³. Seleno-methionine (Se-Met)-substituted protein was obtained from *E. coli* grown in M9 minimal medium using the methionine pathway inhibition approach²⁴, and purified as described for the native protein. HypSc (GenBank accession number AB187170) was subcloned from *Streptomyces coelicolor* A3(2) genomic DNA using the forward primer 5'-GGGGGGCCATGGCCCTACTGG TAGAACGACGG-3' (*Nco*I site underlined) and the reverse primer 5'-GGGGGGGATCTCCAGCGTTCCAGTACCGC-3' (*Bam*HI site underlined), inserted into the *Nco*I and *Bam*HI sites of pHis8, overexpressed in *E. coli* as an octahistidine-tagged protein and purified by Ni^{2+} -chelation chromatography and gel filtration chromatography, as described²³.

Crystallization and data collection. Crystals were obtained by vapour diffusion at 4 °C. Two-microlitre hanging drops containing a 1:1 mixture of 15 mg ml⁻¹ protein with crystallization buffer (28% (w/v) PEG 4000, 0.3 M magnesium nitrate, 2 mM DL-dithiothreitol (DTT), 0.1 M TAPS, pH 8.5) equilibrated over a 500 μ l reservoir of the same solution produced small diffracting crystals overnight. Larger crystals were obtained by macro-seeding into the same conditions. Crystals were stabilized by soaking briefly in a cryoprotectant solution (30% (w/v) PEG 4000, 15% (v/v) glycerol, 0.3 M magnesium nitrate, 2 mM DTT, 0.1 M TAPS, pH 8.5), and flash frozen in liquid nitrogen before data collection. Orf2 crystals belong to the $P2_12_12$ space group with average unit cell dimensions of $a = 71$ Å, $b = 92$ Å, $c = 48$ Å, $\alpha = \beta = \gamma = 90^\circ$, contain one monomer per asymmetric unit and have a solvent content of 45%. Se-Met-substituted crystals were obtained as described for native crystals. Substrate and substrate analogue complexes were obtained by soaking Orf2 crystals in stabilization solutions containing 5 mM GPP, or 10 mM GSPP and 40 mM 1,6-DHN, or 10 mM GSPP and 10 mM flaviolin (GPP and GSPP were purchased from Echelon Biosciences).

Structure determination and refinement. A multi-wavelength anomalous dispersion (MAD) data set was collected at the selenium edge on a Se-Met

Orf2 crystal at the Brookhaven National Laboratory (BNL) on beamline X8C. Data were processed with HKL2000 (ref. 25) and reduced to a unique set of indexed intensities to a resolution of 1.6 Å. Single-wavelength data sets were collected at the Salk Institute for Biological Studies, BNL (beamline X6A), the European Synchrotron Radiation Facility (ESRF, beamline FIP/BM30A) and the Stanford Synchrotron Radiation Laboratory (SSRL, beamline 9.1) on substrate and substrate analogue complexes (Supplementary Table S2). Phasing, density modification and automatic model building were carried out with the program suite Solve/Resolve²⁶ using seven identified Se sites. Additional rounds of building and refinement were carried out with O²⁷ and CNS²⁸, respectively. Subsequent complexes were solved by molecular replacement with AMoRe²⁹.

Prenylation assays and product identification. The reaction conditions for prenylation of 1,6-DHN using either Orf2 or HypSc consisted of 50 mM HEPES (pH 7.5), either no MgCl₂ (minus) or 5 mM MgCl₂ (plus), 5 mM 1,6-DHN, and 5 mM DMAPP, GPP or FPP in a final volume of 20 µl. Reactions were initiated with 20 µg of Orf2 or 20 µg HypSc. After incubation at 25 °C for 4 h (Orf2) or overnight (HypSc), reactions were dried and spotted on a silica gel TLC plate. TLC plates were developed with a chloroform/methanol (20:1) solvent mixture. 1,6-DHN and reaction products were detected at 254 nm. The chemical analyses of the two HPLC-purified products obtained from Orf2 incubations were accomplished by MS and ¹H NMR (Supplementary Data). Reactions measuring the activity of Orf2 against various synthetic and natural aromatic molecules (see Fig. 2b) consisted of 50 mM HEPES (pH 7.5), 5 mM MgCl₂, 0.1 mM GPP, 0.009 mM [¹⁴C]GPP, 0.1 mM each aromatic substrate, 30 µg of Orf2 in a final volume of 20 µl. After incubation at 25 °C for 6 h, the mixtures were dried, spotted on a silica gel TLC plate and developed with a chloroform/methanol (15:1) solvent mixture. Products were detected using a [¹⁴C] imaging plate (Fuji Photo Film).

Database searches. Searches were performed with PSI-BLAST and VAST (<http://www.ncbi.nlm.nih.gov>), SSM and DALI (<http://www.ebi.ac.uk/msd-srv/ssm>), CE (<http://cl.sdsc.edu/ce.html>) and DEJAVU (<http://portray.bmc.uu.se/>), available through the Protein Data Bank (<http://www.rcsb.org/pdb/>), the Structural Classification of Proteins (SCOP; <http://scop.mrc-lmb.cam.ac.uk/scop>), and the CATH Protein structure classification (<http://www.biochem.ucl.ac.uk/bsm/cath>) websites.

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Supplementary Information is linked to the online version of the paper at www.nature.com/nature.

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Author Information The atomic coordinates and structure factors of Orf2 in complex with TAPS, Mg²⁺-GPP, Mg²⁺-GSPP-1,6-DHN and Mg²⁺-GSPP-flaviolin have been deposited in the Protein Data Bank under accession codes 1ZDY, 1ZCW, 1ZB6 and 1ZDW, respectively. Reprints and permissions information is available at npg.nature.com/reprintsandpermissions. The authors declare no competing financial interests. Correspondence and requests for materials should be addressed to S.B.R. (richard@salk.edu).

Getting down to details

MicroRNAs that tweak gene expression, single nucleotide polymorphisms in population genetics, and individual genome sequencing: Caitlin Smith takes a look at three fast-moving areas in genomics.

Over the past few years, genomics researchers have been getting to grips with a 'new' genome element — microRNA (miRNA). Although a small number of miRNAs have been familiar to developmental biologists for years, a plethora of miRNAs has recently been discovered in animal and plant genomes. More than 200 miRNAs have been identified in mammalian genomes, but their functions mostly remain a mystery. Silencing gene expression in a similar way to small interfering RNAs (see *Nature* **431**, 350; 2004), mammalian miRNAs are implicated in the control of cell and tissue differentiation, apoptosis, insulin secretion, fat metabolism and cancer.

"We are now aware that there is substantially more transcription from human chromosomes than can be accounted for by the current predictions of human genes," says Frank Slack at Yale University, New Haven, Connecticut. Slack is studying the apparent involvement of the miRNA *let-7* in lung cancer and the implications of its ability to suppress translation of the oncogene *RAS*. "Many miRNAs are mapping to disease loci where previously a gene was not found," he says.

miRNA research is a typical microcosm of

the variety of disciplines and techniques that are required to make sense of the genome — computational biology, bioinformatics and comparative genomics to predict candidate miRNAs, followed by classic 'wet biology' to validate the candidates and study their expression and function. And as more labs are gearing up to study miRNAs, commercial products tailored to help them are coming onto the market.

The technical problems of detecting miRNAs in total cellular RNA stem from their small size and often low abundance. Produced

from a larger precursor molecule, mature miRNAs are RNA hairpins of 17–23 nucleotides, which bind to complementary sequences in their target messenger RNAs (mRNAs) and prevent translation. The general techniques for detecting and isolating miRNAs



Micro solution: Ambion's flashPage isolates small RNAs.

from cellular RNA are those used for other small RNAs. A first step could be spin-column fractionation of RNA to remove larger RNAs, using columns such as the Amicon YM-100 from Millipore of Bedford, Massachusetts, which will remove RNAs of more than 75 bases, or the PureLink miRNA isolation kit from Invitrogen of Carlsbad, California, with a 200-nucleotide limit. Qiagen of Valencia, California, has a small RNA protocol for their widely used RNeasy system, which will remove RNAs of more than 200 bases from total cellular

RNA. To get even closer to mature miRNA length, RNA specialists Ambion of Austin, Texas, sells a flashPage, a gel-based fractionation machine for the rapid isolation of small nucleic acids of around 40 bases.

After initial preparation, specific miRNAs

BIG TASKS FOR SMALL MOLECULES

Having helped to identify many miRNAs, Christopher Burge and his colleagues at the Massachusetts Institute of Technology are one of many teams now tackling an even bigger job — to find out which genes are regulated by the known miRNAs, and how they fit into physiological pathways.

Finding targets begins computationally, using the TargetScan algorithms developed by Benjamin Lewis working with Burge and with David Bartel at the Whitehead Institute for Biomedical Research in Cambridge, Massachusetts. These algorithms "rely on evolutionary conservation of segments complementary to the microRNA 'seed' region in the 3' untranslated regions of orthologous genes from multiple vertebrate organisms", says Burge. The seed region, six or seven bases at the 5' end of the miRNA, is thought to be key to



James Carrington: taking a systems look at miRNA.

specifying which genes an miRNA will regulate. Targets have been verified in Bartel's lab using a dual luciferase reporter system, which measures the effect of predicted miRNA interaction sites on protein production in cultured human cells. In a computational analysis published earlier this year, Lewis, Burge and Bartel estimated that more than a third of our genes might be regulated by miRNAs.

The task will be complicated by the fact that an miRNA may regulate as many as 200 genes, according to a computational study by Nikolaus Rajewsky and his colleagues at New York University and Rockefeller University, using their PicTar algorithm to identify miRNA targets. Other software for miRNA target prediction includes miRANDA from Anton Enright and his colleagues at the Memorial Sloan-Kettering Cancer Center in New York and DIANA-microT from Artemis Hatzigeorgiou and Axel Bernal at the University of Pennsylvania, Philadelphia.

Frank Slack's team at Yale University uses *in situ* hybridization, northern blots and fluorescent protein fusions to find when and where miRNAs and their targets are expressed. "We use genetics and RNA interference to reduce the expression of potential targets to see if we suppress the effects of

a mutation in the corresponding miRNA, and use reporter gene assays to test if the miRNA-complementary sites function in gene regulation," he says.

"The classic tools of developmental biology and physiology are needed to correlate miRNA expression and targeting to biological function," agrees James Carrington at Oregon State University, Corvallis, who is looking at pathways regulated by miRNAs in *Arabidopsis*. "miRNA sensors involving miRNA target sites within gene constructs expressing a fluorescent protein are quite useful in understanding spatial and temporal miRNA expression and activity patterns," he says. But to address the question of how miRNAs integrate with cellular pathways, "the more quantitative approaches using the tools of systems biology and computational analysis are the trend in this lab", he says. C.S.

in the sample can be detected by techniques such as northern analysis, PCR and microarrays. But how do you know what you're looking for? Much of the groundwork in miRNA identification has been laid by large-scale genomics projects that used computational techniques to predict miRNA genes followed by cloning and validation of the predicted sequence. The public miRNA registry currently holds around 1,650 entries for published predicted miRNAs. The big projects now under way are to determine which genes the miRNAs are targeting (see 'Big tasks for small molecules', page 991).

Northern analysis is still the standard for detecting and quantifying miRNA expression. "Northern blotting, even if time consuming, is by far the best technique to study miRNA expression because of its sensitivity and quantitativeness," says Jiahui Han at the Scripps Research Institute, La Jolla, California, who is looking at the mechanisms by which miRNAs affect the stability of their target mRNAs. "Primer extension has the advantage of being quicker but, unfortunately, is less quantitative," he says.

Integrated DNA Technologies (IDT) of Coralville, Iowa, sells miRNA tools to increase the sensitivity of northern analysis. Its StarFire kit for probe labelling makes labels composed of 10 ³²P- α -dATPs rather than the more usual single ³²P- γ -ATP. "We use a special template and reaction conditions that give a 10-base tail with almost no heterogeneity," explains Mark Behlke, vice president of molecular genetics at IDT, "so all probe molecules

are the same — it is very different from other tailing procedures." Manufacturers are also gearing up to make miRNA-specific probes; miRCURY LNA (locked nucleic acid) detection probes for all known miRNAs are available from Exiqon of Vedbaek, Denmark, for example, and can be used for *in situ* hybridization, northern analysis, PCR and gene knockdown.

Another choice for miRNA detection is Ambion's mirVana miRNA detection kit. Ambion claims that its assay is 100–500 times more sensitive than northern analysis, as the radiolabelled probes are hybridized in solution instead of on a membrane as in northern blotting. The company claims that this method gives the researcher a better shot at detecting and quantifying low-abundance miRNAs because the probe and target have more opportunities to bind when in solution.

Taking the PCR road, Applied Biosystems of Foster City, California, is soon to launch a new TaqMan microRNA assay for miRNA detection and quantitation, which the company claims will detect only mature miRNAs and not precursors. According to Marcum Bell, product manager of gene-expression assays at Applied Biosystems, the assay "uses specific stem-looped primers for reverse transcription of the mature miRNA, followed by quantitative real-time PCR." A claimed advantage of the new assay is its wide dynamic range of up to 7 log units, enabling detection of both low- and high-abundance miRNAs.

For an alternative to PCR-based miRNA assays, US Genomics of Woburn, Massachu-

setts, recently unveiled its Trilogy 2020 Single Molecule Analyzer for the high-throughput detection and quantitation of single molecules of nucleic acid without amplification. The Trilogy 2020 can be used along with the company's Direct miRNA Assays for miRNA work. The assay includes two fluorescently tagged probes (tags can be red, blue or green) that are designed to hybridize to the miRNA of interest. Specificity relies on the very high likelihood that only the target miRNA will hybridize to both probes. After hybridization,

US GENOMICS



The Trilogy Single Molecule Analyzer can be used for miRNA detection.

GENOTYPING GETS UP TO SPEED

At the high-throughput end of multiplex SNP genotyping, Illumina of San Diego, California, is currently beta testing the Sentrix Human-1 BeadChip, containing more than 100,000 SNPs, nearly 30,000 of which are located in genes, with another 40,000 within 10 kb of genes. The company is developing BeadChips containing 250,000 and 500,000 SNPs for release next year, which will make it possible to genotype 1 million SNPs on just a pair of chips.

Using a different approach to SNP genotyping, the LightType Genotyping System from Roche Applied Science of Indianapolis, Indiana, is designed for the heavy-duty end of the market, where thousands of samples may have to be genotyped each day. After PCR amplification of genomic samples in 96- or 384-well plates using a standard thermal cycler, plates are transferred directly to the LightType and genotyped within

10 minutes, using the melting points of fluorescently labelled probes hybridized with the SNPs as the detection system. Probe-target complexes with different melting points reflect the presence of different alleles, and show up as allele-specific peaks in the melting curves. Because many samples can be tested simultaneously, "the LightType instrument is mainly

used for SNP genotyping, in particular for disease association studies," says Burkhard Ziebolz of science communications at Roche Diagnostics in Mannheim, Germany.

The Luminex xMAP platform for multiplex genotyping is used by several genetic diagnostics service companies, including TmBioscience of Toronto, Ontario,

which has developed the first Food and Drug Administration-approved multiplexed test for cystic fibrosis mutations, and Tepnel LifeCodes of Manchester, UK, whose speciality is HLA DNA typing.

For less-intensive SNP detection, the READIT SNP genotyping system from Promega of Madison, Wisconsin, can be scaled up or down. It uses the company's READase-mediated destabilization of perfectly matched probe-target complexes coupled with a luciferase reporter assay for the ATP generated. With appropriately designed probes, the system can detect SNPs, insertions, deletions and chromosomal translocation, and can estimate allele frequency and carry out allele-correlation studies. And PerkinElmer of Boston, Massachusetts, have SNP detection kits in their established AcycloPrime range.

TOM MERCE/CLEVELAND CLINIC



Roche's LightType speeds up high-throughput genotyping.

the sample is moved by microfluidics through a glass capillary, where lasers excite the probes at different wavelengths. A target miRNA molecule is counted when photons of both colours are emitted simultaneously.

Both conventional microarrays and bead-based multiplex assay platforms such as xMAP from Luminex of Austin, Texas, can be used to study miRNA expression, and a number of companies offer miRNA products designed for use with microarray systems. PerkinElmer of Boston, Massachusetts, sells a MICROMAX ASAP labeling kit for miRNAs for detection by the tyramide signal amplification (TSA) method, while the Array 900miRNA labeling kits from Genisphere of Hatfield, Pennsylvania, are designed to label miRNAs and other small RNAs with Genisphere's 3DNA dendrimers. If you don't want to do it yourself, companies such as molecular diagnostics specialists Genaco of Huntsville, Alabama, and genetic services company DNAVision of Charleroi, Belgium, offer miRNA expression profiling and quantitation using Luminex xMAP technology. LC Sciences of Houston, Texas and Icoria of Research Triangle Park offer microarray-based miRNA detection covering all miRNAs currently listed in the public miRNA registry.

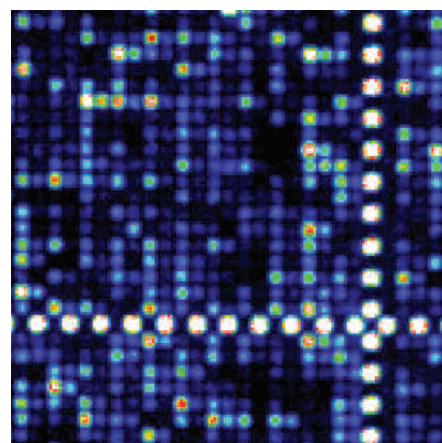
Differences matter

If miRNAs are the new kid on the block in genomics, single nucleotide polymorphisms (SNPs) are already big business (see 'Genotyping gets up to speed', opposite). Your DNA is 99.9% identical to that of another unrelated

human, but it is that last 0.1% that interests researchers. Much of the difference is made up of SNPs, which are sites in DNA that differ by a single base. Groups of SNPs close to one another on a chromosome are called blocks, and are usually inherited together as a haplotype, thus providing a convenient marker for the other genes in the block. The HapMap project, run by the International HapMap Consortium, aims to create a map of these haplotypes and their SNP tags for future research (see 'SNPs and human disease', below).

Using SNP tags, scientists can more efficiently scan an individual's genome for association with phenotypes, such as disease susceptibility, or reactions to drugs or vaccines. Launched in October 2002, the HapMap project hoped to complete the mapping of one million SNP markers by September 2005. When it achieved this goal months ahead of schedule, the consortium announced this February that it will step up its efforts in the second phase to create an improved map that is five times denser than the first draft. This will enable geneticists to zero in on smaller areas of the genome, locating targets more precisely by using more SNP signposts, increasing coverage from one SNP every 3,000 bases (at present) to one every 600 bases.

Vital to phase 2 is Perlegen Sciences of Mountain View, California, which is testing 4.6 million SNPs from public databases for addition to the HapMap. Last September, funded by a grant from the US National Human Genome Research Institute, Perlegen



Patterns of life: DNA bound to a small region of an Affymetrix 100K GeneChip Set.

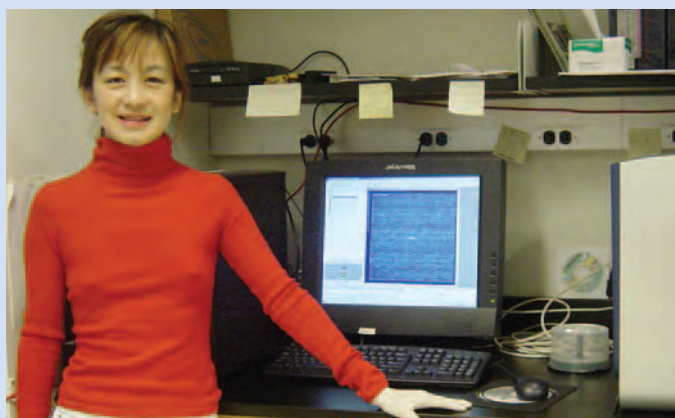
began using high-density oligonucleotide array technology from Affymetrix of Santa Clara, California, to genotype more than 2.25 million unique SNPs from the four HapMap study populations. Perlegen's original goal was to catalogue 600 million genotypes; the new funding in phase 2 should result in more than a billion. The human genome is thought to contain about 10 million SNPs, but not all of these will be useful predictors of disease.

David Cox and his colleagues at Perlegen aim to narrow the field. They have analysed the most common SNPs by mapping 1.5 million SNPs for 71 people from three different ethnic groups: European American, African American and Han Chinese American. The aim is to obtain a high-quality subset of SNPs

SNPs AND HUMAN DISEASE

One goal of the HapMap project is to help researchers find SNPs associated with human disease.

Josephine Hoh at Yale University's School of Public Health and colleagues at Rockefeller University, New York, and the National Eye Institute in Bethesda, have identified an SNP associated with age-related macular degeneration (AMD), a major cause of blindness in people over 60. The SNP is in the gene for complement factor H, leading to a tyrosine to histidine mutation. The researchers studied 96 patients with AMD and 50 healthy controls, and measured the frequency of over 116,000 SNPs in each group. "For the initial screen, we used Affymetrix's set of 100K SNP chips," says team member Robert Klein. "To identify the putative causal mutation, we used PCR to amplify each exon in a number of samples and then resequenced to find all variants in the exons."



Josephine Hoh uses SNP arrays to find mutations associated with disease.

Hoh and her colleagues found that caucasian patients with AMD are at least four times more likely than usual to have this SNP. How the change causes AMD is not yet known, and one of the next directions for her lab "is to figure out the functional mechanism of complement factor H in the pathogenesis of AMD", says Hoh.

There are a few clues. The amino-acid change lies in a part of factor H that interacts with C-reactive protein and heparin, both known to be associated with AMD. And factor H is known to regulate components of the immune system that are found in drusen, fatty deposits that accumulate in the macula with age. In people

with AMD, the drusen are larger and more numerous, killing cells needed to nourish adjacent retinal photoreceptors, which eventually results in loss of sight.

SNP mapping is also underway in animal models of human disease. Kent Hunter at the National Cancer Institute (NCI) in Bethesda, Maryland, uses SNPs to look for cancer-modifying genes in mice. "Ultimately, we hope to identify the particular polymorphic gene or genes that modulate metastatic efficiency," he says. Maxwell Lee at the NCI is interested in how genetic variation determines gene expression and phenotypes in human cancer and uses SNPs to search for epigenetic markers. "We need to understand more dynamic aspects of the genome including interactions between SNPs and other downstream targets such as chromatin, DNA methylation and gene expression," he says. C.S.

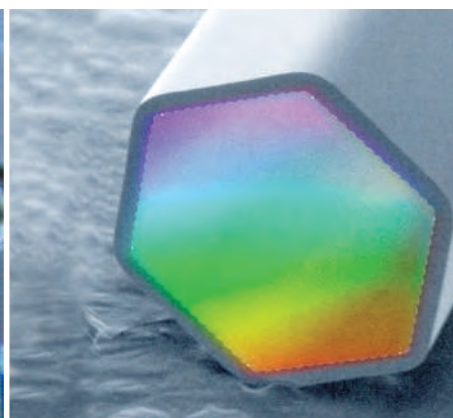
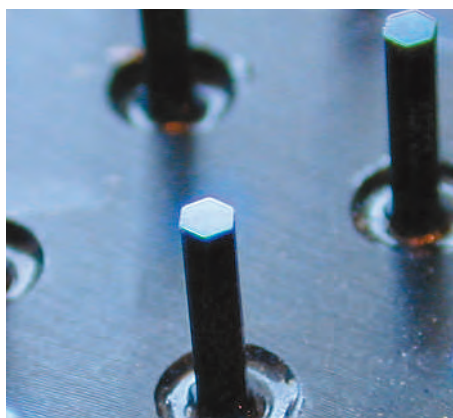
for disease prediction, and to make these subsets more useful by learning more about the frequencies of alleles and how they are correlated with one another.

The \$1,000 genome?

As Illumina closes in on the million-SNP assay (see 'Genotyping gets up to speed', page 992), others are striving for the \$1,000 genome — a quick and cheap method of sequencing individual genomes. This somewhat arbitrary goal has caught the fancy of scientists and is being competitively pursued by companies — fuelled in part by a \$500,000 cash prize offered by the J. Craig Venter Science Foundation to whoever gets there first.

For this goal to become reality, a new method must replace the much-loved Sanger method and its offspring. The leading alternative is single-molecule-based sequencing, also known as sequencing by synthesis. VisiGen Biotechnologies in Houston, Texas, uses this method with single-pair fluorescence resonance energy transfer (spFRET) as the detection technology. The donor fluorophore is attached to a DNA polymerase that sits on the template, while a colour-coded acceptor fluorophore is attached to the gamma-phosphate of a nucleoside triphosphate. When the nucleotide is incorporated into DNA, the donor fluorophore stimulates the acceptor to emit a characteristic fluorescent signal (measured as emission wavelength and intensity) that indicates its base identity — each base is a different colour.

"The donor fluorophore acts as a punctuation mark between nucleotide incorporation events," explains Susan Hardin, president and chief executive of VisiGen. Massively parallel arrays of these reactions produce a high-throughput sequencing system without the need for electrophoresis, cloning or PCR. Hardin points out that using a donor label on



A Sentrix array matrix (left) used for genotyping and one of its fiber bundles (right).

an immobilized polymerase "minimizes background, increases consistency of the signal during the extension, and increases read length." The advantage of labeling the nucleotide on its terminal gamma phosphate is that the fluorophore does not become part of the nascent DNA strand.

The sequencing by synthesis method of Solexa, based in Little Chesterford, UK, (which recently merged with Lynx Therapeutics of Hayward, California) differs from that of VisiGen mainly in the detection method. In the Solexa technology, the different types of fluorescently labelled nucleotide incorporated into the DNA strand are detected by excitation with an external light source. The cycle of nucleotide incorporation, detection and identification is repeated about 25 times to read the first 25 bases in each oligonucleotide in an array of millions of single-stranded genomic DNA fragments.

According to Simon Bennett, Solexa's business development director, one advantage of the company's system is the small sample volume required — only a few picograms. "Biobanks may need to seriously reconsider how to collect and store samples, and may wish to explore cheaper options," says Bennett. "With the emerging technologies the cost of analysing samples, and how much sample is needed for each subject, is almost certain to reduce dramatically." He also remarks that the short sequence read lengths of 25–30 nucleotides in Solexa's method may allow a degraded sample to be resequenced, rendering a previously useless sample once again viable.

A stab at the \$1,000 genome is also being taken by 454 Life Sciences, a subsidiary of Curagen in Branford, Connecticut. The 454 whole-genome sequencing system, a prototype of which was recently installed at the Broad Institute in Cambridge, Massachusetts, uses the patented light-emitting 'pyrosequencing' chemistry developed by Pyrosequencing of Uppsala, Sweden, (now renamed Biotage after its recent takeover of that company) and microfluidics nanotechnology developed by 454. The pyrosequencing technique was exclusively licensed to 454 in 2003 for the purposes of developing it for whole-genome

sequencing. Biotage retains the rights to use the technology in its gene-analysis products, such as the PyroMark range of genetic tests launched earlier this year. Last month, Roche Applied Science signed an exclusive licence with 454 to develop and sell the 454 whole-genome sequencer.

So when can we expect the first \$1,000 genome? "2010 to 2012," says Hardin. Bennett is less definite, but thinks that Solexa will be offering a \$1,000 genome product before 2016, and "probably before the end of this decade."

Future prospects

Looking to what's next on the genomics agenda, Carrington settles for understanding the mechanisms of genome evolution and adaptation, especially the evolution of new functions. He cites the recent discovery of possible "multigenerational sequence caches to restore lost information from a genome," in which plants have been shown to inherit DNA sequences not apparently present in parental genomes yet found in previous generations; one explanation might be caches of RNA. "Understanding the mechanisms of formation and adaptation of new miRNA genes, and more broadly, the derivation and deployment of small RNA-based regulation at the transcriptional and post-transcriptional levels, represent a major set of questions under the umbrella of genome evolution," he adds.

On the biomedical front, Hardin points out that geneticists will soon be facing serious ethical considerations. "Who should have access to an individual's genome sequence?" she asks. "What should one be told about (potential) defects in one's genome sequence? When? How can we best safeguard the privacy of genome-sequence information?" Given the rapid advance of genomics, scientists will be having to answer these questions sooner than they might have thought. ■

Caitlin Smith is a science writer based in Portland, Oregon.

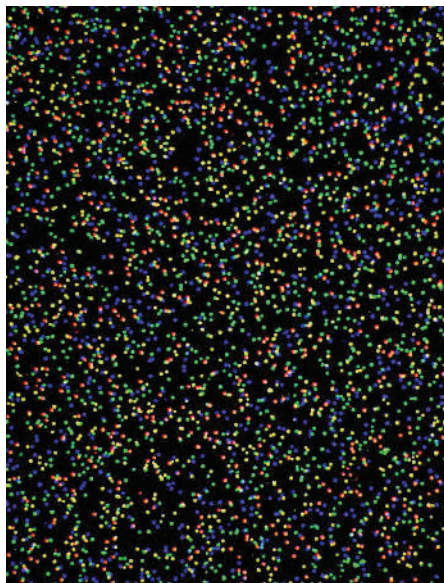
miRNA Registry

♦ www.sanger.ac.uk/Software/Rfam/mirna/index.shtml

International HapMap Consortium

♦ www.hapmap.org

SOLEXA LTD



Light fantastic: the first cycle in a round of sequencing by Solexa's method.

COMPANY	PRODUCTS/ACTIVITY	LOCATION	URL
MicroRNA			
Ambion	mirVana range for miRNA isolation and detection, flashPage for isolation of small RNAs	Austin, Texas	www.ambion.com
DNAVision	Pharmacogenomics, gene-expression analysis, miRNA quantitation analysis	Charleroi, Belgium	www.dnavision.be
Exiqon	Microarray slides and chips, LNA oligonucleotides, miRCURY probes for miRNA	Vedbaek, Denmark	www.exiqon.com
Genaco	mirMASA platform for multiplex miRNA expression profiling, tumour miRNA database	Huntsville, Alabama	gene.genaco.com
Genisphere	3DNA probes, including miRNA probes, RNA amplification	Hatfield, Pennsylvania	www.genisphere.com
Icoria	Paradigm Array range includes human MirChip miRNA array	Research Triangle Park, North Carolina	www.icoria.com
Integrated DNA Technologies	Custom oligonucleotides, reagents for cloning and radioactive probe labelling	Coralville, Iowa	www.idtdna.com
Invitrogen	Kits and reagents for cloning, genomics, proteomics, molecular and cell biology, PureLink miRNA isolation kit for small RNAs, Molecular Probes products	Carlsbad, California	www.invitrogen.com
LC Sciences	Oligonucleotides, speciality microarrays, miRNA detection arrays	Houston, Texas	www.lcsciences.com
PerkinElmer Life Sciences	Laboratory instruments and equipment, MICROMAX ASAP miRNA labelling kit for MICROMAX human DNA microarrays, SNP detection kits	Boston, Massachusetts	lifesciences.perkinelmer.com
Rosetta Genomics	microRNA discovery, validation, bioinformatics and microarray development	Rehovot, Israel	www.rosettagenomics.com
US Genomics	Direct miRNA Assay for the Trilogy 2020 Single Molecule Analyzer	Woburn, Massachusetts	www.usgenomics.com
Genotyping and mutation detection			
Affymetrix	DNA microarrays, SNP genotyping microarrays, scanners, RNA amplification kits	Santa Clara, California	www.affymetrix.com
Agencourt Bioscience Corporation	Instruments and products for DNA and RNA purification, SNP discovery services	Beverly, Massachusetts	www.agencourt.com
Applied Biosystems	Instruments for molecular biology, genomics and proteomics, SNaPshot multiplex kit, SNplex genotyping system, TaqMan SNP genotyping assays	Foster City, California	home.appliedbiosystems.com
Asper Biotech	Genorama microarray system, genetic testing	Tartu, Estonia	www.asperbio.com
Beckman Coulter	Automation for molecular biology, biochemistry, genomics and proteomics, GenomeLab SNPstream system for high-throughput genotyping	Fullerton, California	www.beckmancoulter.com
BioCat	Microarrays, SNP genotyping kits and services	Heidelberg, Germany	www.biocat.de
BioCytex	SNP assay development	Marseille, France	www.biocytex.com
BioServe	Genotyping services	Laurel, Maryland	www.bioserve.com
Bio-Rad	Products, instruments and software for life-sciences research, DCode Universal Mutation Detection System for gel-based genotyping	Hercules, California	www.bio-rad.com
Biotage	Pyrosequencing DNA sequencing, PyroMark tests for disease alleles, chromatography	Uppsala, Sweden	www.biotage.com
BioVentures	Cytochrome P polymorphism detection, DNA sequencing services	Murfreesboro, Tennessee	www.bioventures.com
Brucker Daltonics	GenoTools 1.1 for SNP genotyping by mass spectrometry	Billerica, Massachusetts	www.bdal.com
Chemicon	Nucleic acids, reagents and kits for RNA expression, SNP genotyping system	Temecula, California	www.chemicon.com
deCODE Genetics	Pharmacogenomics services, high-throughput PCR-based microsatellite genotyping	Reykjavik, Iceland	www.decode.com
DxS	Genetic analysis services, genotyping using Scorpions platform for PCR	Manchester, UK	www.dxs-genotyping.com
Genaissance	Genotyping and genetic analysis services	New Haven, Connecticut	www.genaissance.com
Genes 4-U	Ready-to-use ToolSets for mutation detection using the LightCycler	Zurich, Switzerland	www.genes-4u.com
Genome Therapeutics	Services for genomics including SNP discovery, validation and screening	Waltham, Massachusetts	www.genomecorp.com
Genometrix	Genotyping, gene-expression and bioinformatics services	The Woodlands, Texas	www.genometrix.com
Illumina	Sentrix Array Matrix and BeadChip platforms for genotyping, oligonucleotide synthesis, SNP genotyping services	San Diego, California	www.illumina.com
Integrage	GenomeHIP (Genome Hybrid Identity Profiling) gene-mapping technology	Evry, France	www.integrage.com
IT Biochem	Probes, primers and reagents for LightCycler and Idaho Technology thermal cyclers	Salt Lake City, Utah	www.idahotech.com/itbiochem
Lambda	Biochips for genotyping and SNP detection	Freistadt, Austria	www.lambda.at
LGC	Pharmacogenetic diagnostics, consultancy and research service	Teddington, UK	www.lgc.co.uk
Luminex Corporation	xMAP platform for microbead-based multiplex assays	Austin, Texas	www.luminexcorp.com
Marligen Biosciences	SNP genotyping systems and services, kits for Y-SNP identification, mitochondrial DNA	Ijamsville, Maryland	www.marligen.com
MiraiBio	Microarray systems and equipment, MasterPlex GT genotyping software	Alameda, California	www.mirai.bio.com
Myriad Genetics	Genomics-based drug discovery and predictive genetic testing	Salt Lake City, Utah	www.myriad.com
Nanogen	NanoChip automated workstation for SNP and STR analysis	San Diego, California	www.nanogen.com
NimbleGen Systems	ENCODE microarray services for ChIP-chip analysis, custom microarray services	Madison, Wisconsin	www.nimblegen.com
Orchid BioSciences	Array-based SNP genotyping platforms, genotyping services	Princeton, New Jersey	www.orchid.com
Panomics	SNPCapture kits for mutations in specific transcription factor and cytokine genes	Redwood City, California	www.panomics.com
ParAllele Bioscience	MegAllele products for custom multiplex SNP genotyping, genome variation scanning	San Francisco, California	www.parallelebio.com
Perlegen Sciences	Microarray-based SNP genotyping	Mountain View, California	www.perlegen.com
Polymorphic DNA Technologies	SNP discovery services, SNP detection kits	Alameda, California	www.polymorphicdna.com
Promega	Reagents and kits for life-sciences research, READ-IT system for SNP genotyping	Madison, Wisconsin	www.promega.com
R&D Systems	cDNA expression kits, mRNA quantitation kits, primer pairs, mutation detection kit	Minneapolis, Minnesota	www.RnDSystems.com
Roche Applied Science	Equipment and consumables for life-sciences research, MagNA Pure nucleic acid purification systems, LightCycler for PCR, LightTyper for automated SNP genotyping	Indianapolis, Indiana	www.roche-applied-science.com
SeqWright	DNA sequencing and genomics services, SNP genotyping	Houston, Texas	www.seqwright.com
Sequenom	MassArray for high-throughput genotyping, SNP discovery, allele-frequency analysis	San Diego, California	www.sequenom.com
SpectruMedix	Instruments for DNA sequencing, GenoSpectrum genotyping software	State College, Pennsylvania	www.spectrumedix.com
Tepnel Lifecodes	LifeMatch HLA DNA typing system; Elucigene products for mutation analysis	Manchester, UK	www.tepnellifecodes.com
Third Wave Technologies	Invader RNA assays and genotyping kits	Madison, Wisconsin	www.twt.com
TmBioscience	Genetic testing services, Tag-It multiplex genotyping tests	Toronto, Canada	www.tmbioscience.com

COMPANY	PRODUCTS/ACTIVITY	LOCATION	URL
Transgenomic	WAVE systems for SNP and mutation detection by chromatography	Omaha, Nebraska	www.transgenomic.com
USB	Products for molecular biology, PCR and mutation discovery	Cleveland, Ohio	www.usbweb.com
Variagenics	Products and services for genotyping and haplotyping for clinical trials	Cambridge Massachusetts	www.variagenics.com
Whole-genome sequencing			
454 Life Sciences	Fast DNA sequencing platform	Branford, Connecticut	www.454.com
Solexa	Fast DNA sequencing platform	Little Chesterford, UK	www.solexa.co.uk
VisiGen Biotechnologies	Fast DNA sequencing platform	Houston, Texas	www.visigenbio.com
General			
Active Motif	Kits and reagents for cell fractionation, nucleic-acid isolation and gene silencing	Carlsbad, California	www.activemotif.com
Agilent	Instruments for genomics and proteomics, DNA microarray kits, RNA amplification	Palo Alto, California	www.agilent.com
Arcturus	Laser-capture microdissection, microgenomics, RNA amplification	Mountain View, California	www.arctur.com
AutoGen	Instruments for nucleic acid extraction	Holliston, Massachusetts	www.autogen.com
BioChain	Products for genomics and proteomics, northern blotting	Hayward, California	www.biocchain.com
Biognostik	Antisense oligonucleotide synthesis kits and services	Göttingen, Germany	www.biognostik.com
Biosearch Technologies	Fluorophores and quenchers for quantitative PCR, Black Hole Quencher	Novato, California	www.biosearchtech.com
Brinkmann	Laboratory instrument suppliers, software, consumables	Westbury, New York	www.brinkmann.com
Capital Genomix	GeneSystem 320 differential gene-expression profiling system	Chantilly, Virginia	www.capitalgenomix.com
Clontech	Reagents for PCR, RNAi expression kits, nucleic-acid purification	Mountain View, California	www.clontech.com
Combimatrix	Customized DNA microarrays, RNAi	Mukilteo, Washington	www.combimatrix.com
Corbett Research	Automated systems for DNA/RNA extraction, real-time PCR	Sydney, New South Wales	www.corbettresearch.com
Dharmacon	siRNA kits and arrays, custom RNAs, large-scale RNA synthesis	Lafayette, Colorado	www.dharmacon.com
EMD Biosciences	Novabiochem, Calbiochem, Novagen and Oncogene Research products	San Diego, California	splash.emdbiosciences.com
EPICENTRE	Products for DNA and RNA purification, DNA sequencing, PCR, RNA amplification	Madison, Wisconsin	www.epibio.com
Eppendorf	Instruments and consumables for life-sciences research, nucleic-acid purification, PCR	Hamburg, Germany	www.eppendorf.com
Eurogentec	Custom oligos, real-time PCR probes and primers, DNA microarrays	Seraing, Belgium	www.eurogentec.be
Evident Technologies	Quantum dot fluorescence labels	Troy, New York	www.evidentech.com
Fluorescentric	Real-time PCR probes, assay design for PCR	Park City, Utah	www.fluorescentric.com
GATC Biotech	High-throughput DNA sequencing and analysis services	Konstanz, Germany	www.gatc-biotech.com
GE Healthcare	MegaBACE sequencing and genotyping systems, CodeLink SNP bioarray	Little Chalfont, UK	www.amersham.co.uk
Genomic Solutions	Automation for genomics and proteomics, software, consumables and services	Ann Arbor, Michigan	www.genomicsolutions.com
GenScript	Kits and services for cell and molecular biology, oligonucleotide synthesis	Piscataway, New Jersey	www.genscript.com
Genra Systems	Versapure automated DNA and RNA purification instruments	Minneapolis, Minnesota	www.genra.com
IBA	Oligonucleotide probes	Göttingen, Germany	www.iba-go.com
KPL	Nucleic-acid labelling and detection kits	Gaithersburg, Maryland	www.kpl.com
Kreatech	Kits for nucleic-acid labelling using non-enzymatic universal linkage	Amsterdam, The Netherlands	www.kreatech.com
Maxim Biotech	PCR probes and kits, custom primer design and synthesis	San Francisco, California	www.maximbio.com
Merck Biosciences	Novabiochem, Calbiochem, Novagen and Oncogene Research products	Darmstadt, Germany	splash.emdbiosciences.com
metabion	LightCycler oligos, custom oligos and probes	Martinsried, Germany	www.metabion.com
Millipore	Equipment and consumables for life-sciences research, Amicon centrifuge filters	Bedford, Massachusetts	www.millipore.com
Mirus	Reagents and kits for nucleic-acid isolation, gene transfer and RNAi	Madison, Wisconsin	www.mirusbio.com
Molecular Beacons	Molecular beacon probes for real-time PCR and <i>in situ</i> RNA detection	Newark, New Jersey	www.molecular-beacons.org
Molecular Research Center	TRI Reagent solutions for nucleic-acid isolation	Cincinnati, Ohio	www.mrcgene.com
Motorola Life Sciences	eSensor BioChip and reader for DNA/RNA detection	Northbrook, Illinois	www.motorola.com/lifesciences
MP Biomedicals	Reagents and biochemicals for life-sciences research	Irvine, California	www.mpbio.com
MWG Biotech	Custom oligonucleotides, siRNA, DNA sequencing services	Ebersberg, Germany	www.mwg-biotech.com
New England Biolabs	Reagents and kits for molecular biology	Beverly, Massachusetts	www.neb.com
Novagen	Reagents and kits for molecular biology	Madison, Wisconsin	www.novagen.com
NuGen Technologies	Ovation RNA amplification process	San Carlos, California	www.nugenttechnologies.com
OligoEngine	Custom oligonucleotides	Seattle, Washington	www.oligoengine.com
Oligos Etc	Oligonucleotides, antisense oligos	Wilsonville, Oregon	www.oligosetc.com
Open Biosystems	Clones, RNAi, chromosome paints, mouse shRNA libraries	Huntsville, Alabama	www.openbiosystems.com
Pierce Biotechnology	Components and consumables for molecular biology	Rockford, Illinois	www.piercenet.com
Proteodyne	BioCube automated systems for genomics and proteomics	Windsor, Connecticut	www.proteodyne.com
Qiagen	Reagents, kits and instruments for the life sciences, LiquiChip for multiplex assays	Valencia, California	www.qiagen.com
QuantumDot Corporation	Nanocrystalline fluorescent tags, Mosaic system for multiplexed mRNA quantification	Hayward, California	www.qdots.com
RNA-TEC	Custom oligonucleotide synthesis	Leuven, Belgium	www.rna-tec.com
RNAworks	StabMRT kits for stabilizing RNA	Montpellier, France	www.rnaworks.net
Sigma-Aldrich	Reagents and biochemicals for life-sciences research	St Louis, Missouri	sigma-aldrich.com
Stratagene	Tools and reagents for molecular biology, genomics and proteomics	La Jolla, California	www.stratagene.com
Synthegen	Modified oligonucleotides, nucleic-acid probes	Houston, Texas	www.synthegen.com
TaKaRa Bio	Reagents, kits and services for life-sciences research, PCR enzymes, DNA microarrays	Shiga, Japan	www.takara-bio.co.jp/english
Thermo Electron Corporation	Instruments and reagents for molecular biology, oligonucleotides	Waltham, Massachusetts	www.thermo.com
TIB MOLBIOL	Oligonucleotides design and synthesis, locked nucleic acids	Berlin, Germany	www.tib-molbiol.de
Upstate	Reagents, antibodies and kits for life-sciences research, siRNA	Charlottesville, Virginia	www.upstate.com
VBC-GENOMICS Research	DNA sequencing, microarray products and services, oligonucleotide synthesis	Vienna, Austria	www.vbc-genomics.com

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A global view

The past year seems to have been a good one for biotechnology, with the sector continuing to expand, according to a report issued this month by analysts Ernst & Young. *Beyond Borders: Global Biotechnology Report 2005* reveals that global biotech employment grew by 5% to 183,820 jobs over the period 2003-04. A disproportionate number of those jobs — 137,400 — are in the United States, which saw its public biotech employment grow by 10% in that period.

The picture is less rosy in Europe, which saw public biotech jobs fall by 21% to 25,640, and in Canada, where the number of jobs dropped from 7,440 to 7,370. Asia-Pacific, on the other hand, saw rapid growth as it moved from having 9,810 jobs to 13,410.

The experts cited in the Ernst & Young report almost all agree that the biotech industry is aiming to cut the costs of drug development. And they also note that companies are more willing than ever to go global. But what will this mean for the future of biotech jobs?

For now, the big centres in the United States — especially Massachusetts and California — will continue to thrive, as

they have a critical mass of infrastructure, investment and personnel. But as companies look to cut costs and outsource parts of their operation, Asia stands to gain — a trend that is apparent beyond biomanufacturing (see *Nature* **433**, 902-903; 2005). And some Asian countries are already beginning to exploit gaps in research and development where politics may be creating obstacles in the United States — by promoting stem-cell science and therapeutic cloning, for instance.

Putting the numbers and trends together, then extrapolating a little, it seems safe to say that the number of jobs in the biotech sector will continue to grow for the next few years. But it is no longer safe to predict that the growth will take place in those places traditionally associated with the field.



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An eastern promise of regeneration

The East Coast corridor of New Jersey, Delaware and Pennsylvania is poised for renewed growth in biomedicine. **Claudia Caruana** investigates.

The science and engineering corridor that stretches through New Jersey, Delaware and Pennsylvania has been somewhat sleepy in recent years. A slew of corporate consolidations and staff cutbacks put the brakes on the region's economy, stifling its growth. But now this sleeping giant is beginning to stir, and job prospects are rising in both industry and academia.

The northeast corridor is home to a number of drug companies, including Merck, Pfizer, Wyeth, Johnson & Johnson and Schering-Plough, all of which have a presence in New Jersey. Many of the region's larger drug firms are looking to expand their research and development (R&D) operations, says Buster Houchins, vice-president of Christian & Timbers of Columbia, Maryland, which recruits for many of the companies in the corridor.

"Today, there are many more 'smaller drugs' in the companies' pipelines, in contrast to just a few potential blockbusters," Houchins says. "These smaller drugs are coming to market soon and, although they may have fewer marketing bucks attached to them, their positioning in the market will mean more R&D personnel will be needed."

In fact, Houchins believes that the recent problems faced by both Merck and Pfizer, which centred on health scares surrounding their COX-2 inhibitor painkillers, could paradoxically generate more jobs

in the region. The companies may well end up seeking new R&D directions, he says, and this could mean a number of new positions in fresh business units. Pfizer, whose consumer healthcare division is based in Morris Plains, New Jersey, is already moving this way. Earlier this month it broke ground on the first building in a \$500-million R&D expansion at its Morris Plains site.

Poised for growth

A general maturing of the biotech sector is also helping to fuel demand for new recruits, notes Julie Kampf, president of executive search firm JBK Associates in Englewood, New Jersey. Last year, her firm saw a 30% rise in healthcare searches, many of which Kampf attributes to biotechnology firms morphing into larger biopharmaceutical companies. "Biopharmas usually hire people with different skill sets, so new people are needed," says Kampf. "And many of these companies are growing significantly in their more traditional businesses, too. Many of the available technical positions we've filled have been in New Jersey."

But it's not just New Jersey that is seeing growth — Delaware is also experiencing a rise in biomedical research and commercial opportunities. Chemical giant DuPont, based in Wilmington, broke ground for its first building in the state in 1802, and has been an important part of Delaware's economy ever since.



Follow the leader: Benjamin Franklin sits in the grounds of the University of Pennsylvania (above), inspiring students and postdocs alike.

PITTSBURGH'S NICHE

Pittsburgh in Pennsylvania is carving a productive niche for itself in biotechnology: regenerative medicine.

"We have strengths here in Pittsburgh that you will not find in other major research centres," says Alan Russell, director of the McGowan Institute for Regenerative Medicine at the University of Pittsburgh. "We focus on that strength."

Regenerative medicine includes tissue engineering, cell therapy and the use of medical devices associated with these techniques.

Russell says that millions of dollars have been invested in his

institute to support research and development, new construction and clinical trials. The influx of both scientists and funds has energized the community, he adds.

The University of Pittsburgh is where the Salk polio vaccine was developed. It now ranks seventh in terms of funding from the National Institutes of Health, and also receives cash from local and regional sources. It is the largest employer in the local healthcare system with 20 hospitals and research centres, and it anticipates hiring an additional 20 researchers specializing in



Healthcare research is a major priority for Pennsylvania.

regenerative medicine over the next three years.

But the university is not alone in the area when it comes to regenerative medicine. It's neighbour, Carnegie Mellon University's Center for Bone Tissue Engineering, is collaborating with Pittsburgh in an attempt to use muscle stem cells to regenerate bone. Jeffrey Hollinger, director of the centre, says that he is planning to hire lab technicians and a cell biologist soon. Although hardly a recruitment stampede, this may signal an upward trend for this burgeoning field.

C.C.



The Fox Chase Cancer Center expects to generate 4,000 new jobs over the next 20 years.

P. COHEN

isn't the only biomedical centre in the state (see 'Pittsburgh's niche', opposite), Philadelphia is seeing a number of medical and research powerhouses expand their presence in the city. The Fox Chase Cancer Center, for instance, is spending \$1-billion over the next 20 years, which by the end will bring in some 4,000 new jobs, many of which will require advanced technical degrees. Similarly, the Wistar Institute, a biomedical research centre on the University of Pennsylvania's campus, is also expanding.

The Wistar was responsible for the world's most widely used rubella vaccine and led work on recombinant-DNA vaccines with a rabies vaccine for animals in the 1980s. Today it is continuing its leading role in the field by collaborating with global pharmaceutical companies. For instance, a rotavirus vaccine developed at the institute is currently undergoing phase III clinical trials through a collaboration with Merck.

Five years ago, there were 26 labs at the institute; now there are 34. Current plans mean that its needs to have an additional eight labs by the end of this decade, says Franklin Hoke, spokesman for the institute.

The Wistar is focusing its growth on four key areas: systems biology (genomics, bioinformatics and proteomics), vaccine development, stem-cell biology and chemical biology (particularly the design of small molecules for drug development). It expects to hire new researchers and postdocs to maintain growth in these areas.

Need for postdocs

At the University of Pennsylvania itself, the number of postdoc positions is steady. But campus recruiting is strong, says a university spokesperson, with postdocs finding themselves in demand with industry as well as academia.

Joyce Gioia, president of management consultants the Herman Group in Greensboro, North Carolina, says that biotechnology and pharmatechnology jobs are growing by leaps and bounds in the northeast corridor. "The particular scientific degree people have doesn't seem to matter," she says. There is a critical shortage of qualified technical people in the corridor, she adds, particularly in biotechnology. "We don't see a downturn in hiring for the foreseeable future," she adds.

Claudia Caruana is a freelance writer based on Long Island, New York.



Although it has no real biopharmaceutical business, it often hires people with skill sets suitable for such operations. "We continue to be interested in the use of advanced materials sciences for medical uses, and hire materials scientists and biomedical engineers at all levels," says John Pierce, the company's director of biosciences and engineering.

DuPont actively recruits from universities around the world. One tool it uses is its Young Professors grant programme, which is designed to provide unrestricted start-up assistance to promising young and untenured research faculty members. To date, more than 500 new scientists have received funding through this programme. "We are looking for talented, experienced scientists from across the market spectrum, including industry, academia and government," says Pierce.

Farther north, in Pennsylvania, Philadelphia is undergoing something of a renaissance. Although it

Damned if you don't

A casual conversation.

Lucy Bergman

I met a guy with a claw. Don't get me wrong — I have nothing against claws, and it wasn't the first one I'd encountered. It's just that he kept talking about it and waving it around. Boarding for the airship was delayed, as usual, so I didn't mind the chat.

He challenged me to guess his age. I gazed at his face, which looked vaguely familiar. The skin — the non-bearded part that I could see — was smooth. Even smoother than mine, which benefits from hours spent every week inside an anti-oxidation rejuvenating chamber. And judging by his, he looked younger than me, I had to admit. But his speech and his expression had the gravity of a much older person. I threw him my puzzled look.

"It's artificially grown, my skin," he told me. "I designed it to be extra thick, for firmness and elasticity. It has natural UV filters and I don't even get the odd blemish."

I was awestruck. I'd read about him.

"Are you Stephen Pflaumbaum?" I asked, mentally pinching myself.

He nodded, and held up the claw for my perusal.

"I've had a lifelong passion to make myself a new hand. The skin and soft tissue part turned out to be easy, but I'm still struggling with the individual bones. I can grow acres of bone, but the hand is very complicated and I will only settle for a perfect replica." Smiling slyly, he added: "And I've been distracted recently by the construction of the dam."

The dam he so casually dropped into conversation is the most ambitious architectural marvel of the twenty-first century, the MedDam at Gibraltar. It is made almost exclusively of living bone, the only material that grows and strengthens under

stress. In the Netherlands they reinforced their dykes with Bucky-wall™, made of carbon nanotubes, but they failed catastrophically two years ago after the most powerful storm surge for a decade. And now the capital city is Maastricht.

Buckywall™ had also bid for the Med-Dam contract but in the end the Mediterranean Council charged with the preservation of the coastline decided to go with Pflaumbaum's plans. His structures are aesthetically appealing as well as being strong and flexible. And once the scaffolding is in place, the high-density bone tissue grows quickly. He'd made a striking demonstration by regrowing one section of the Great Wall in three weeks. Its smooth ivory curve has become a tourist attraction.

A synthetic voice interrupted my thoughts.

"We are sorry to announce that boarding will be delayed by a further 20 minutes. This is due to the late arrival of the incoming airship. We apologize for any inconvenience caused."

I said to Pflaumbaum: "That is exactly the same excuse I hear everyday during my commute! Except it's a ferry."

"Where do you work?" he asked.

"London," I replied. "I'm part of the team redesigning the canal system. In fact, that's why I'm going to Venice. And of course I'd love to visit the old city if I have time. I qualified last month."

At this he brightened, saying, "I'm going to Venice myself. I used to love it as a child. One time I nearly fell into the water after turning a corner because it was so dark. Luckily my mother grabbed me and held

me back, but she nearly ripped out my arm. How I cried and cried until she bought me some gelato. Actually, I didn't even need that as an excuse to get ice cream in those days. I only had to look down at my hand dejectedly and my mother would start pouring out her sympathy. What a horror I was. Plenty of kids had malformed bits then. Now it's all sorted out before birth."

His eyes glanced inadvertently at my right hand. I wanted to withdraw it into my sleeve but refrained. He must have realized because he changed the subject.

"I think we're about to board," he said, nodding at the three gates. Indeed, a crowd had gathered by each door, despite knowing full well that seating is by sections and floors. Did they think we would leave without them?

I turned to Pflaumbaum. "It was a pleasure to meet you. I should like to see your dam one day. I'll have an ice cream in your honour."

He smiled. "Enjoy your trip. You really must visit Old Venice. The light down there can be extraordinary. The water is very clear and last time I followed a school of fish through the ducal palace."

Then leaning in, he added: "You know, I'm going to a conference to discuss whether or not we should pump out some water from the Mediterranean, now that the dam is nearing completion. This might be your last chance to dive in San Marco."

With a wink he went to join the hordes. ■ Lucy Bergman lives in Cambridge, UK, and believes the pen is mightier than the pen.

JACEY

FUTURES